12-2013

Aqueous extracts of Hibiscus sabdariffa as antimicrobials in foods

Kristen Liane Higginbotham
University of Tennessee - Knoxville, khiggin4@utk.edu

To the Graduate Council:

I am submitting herewith a thesis written by Kristen Liane Higginbotham entitled "Aqueous extracts of Hibiscus sabdariffa as antimicrobials in foods." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

Charles N. Stewart Jr., Major Professor

We have read this thesis and recommend its acceptance:

Svetlana Zivanovic, P. Michael Davidson

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

Aqueous extracts of *Hibiscus sabdariffa* as antimicrobials in foods

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Kristen Liane Higginbotham
December 2013

Dedication

This thesis is dedicated to my parents. Without their love and support I wouldn't be where I am today.

Acknowledgements

I would first like to thank Dr. C. Neal Stewart, Jr. for agreeing to be my major professor and providing me with funding, which allowed me to continue the research I had done as an undergraduate student. I would also like to thank Dr. Svetlana Zivanovic for serving as my co-advisor and helping me with anything chemistry related and Dr. P. M. Davidson for serving on my committee and offering helpful advice for the microbiology components of my research.

I especially thank Dr. Kellie Parks Burris. Without her help and support I would not be where I am today. She mentored me throughout the entire process, from starting out as an undergraduate teaching me the basics through my entire Master's program. She helped me and encouraged me with everything I did. I cannot thank her enough.

I would also like to thank my parents, John Higginbotham and Brenda Foley, and boyfriend, Adam Carr, for their never ending love and support. They have encouraged me throughout the entire process.

Abstract

Hibiscus sabdariffa L. is a tropical shrub species cultivated in multiple countries and is mainly produced for its red calyces that are used for a tea beverage. Aqueous, lyophilized extracts of *Hibiscus* were examined for their chemical composition and antimicrobial activity against *Escherichia coli* O157:H7, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Listeria monocytogenes.*

Lyophilized, dialyzed extracts of commercially aquired calyces were examined in microbiological medium and milk at various fat levels for antimicrobial activity against *E. coli* O157:H7 and *S.aureus.* Extracts were either filtered or autoclaved to sterilize and tested in microbiological medium. Autoclaved extracts were more effective in microbiological medium and were subsequently tested in ultrahigh temperature-processed (UHT) milk with various fat percentages. *E. coli* O157:H7 was inactivated after 96 h using 60 mg/ml extracts in all milk fat levels while *S. aureus* was inactivated after 168 h using 40 mg/ml extracts in both skim and whole milk.

Lyophilized, undialyzed extracts were tested as an antimicrobial rinse on hot dogs against *L. monocytogenes* and MRSA. Inoculated hot dogs were rinsed with autoclaved extracts (0, 120 and 240 mg/ml) for 5, 15, 30, or 60 min and stored for 0 or 24 h. Reductions of ca. 3.7 log CFU/g and 5.5 log CFU/g were observed for *L. monocytogenes* and MRSA respectively when rinsed in extracts at 240 mg/ml for 60 min and after 24 h.

Extracts produced from commercial brands were compared to greenhouse grown varieties of *Hibiscus*. Anthocyanin and phenolic contents were determined to compare chemical profiles. Extracts at 60 mg/ml were adjusted to pH 7, autoclaved, and antimicrobial activity was determined against *S. aureus* ATCC 27708. Growth of *S. aureus* was inhibited when grown in the presence of extracts at 60 mg/ml at pH 7. Phenolic content remained similar after autoclaving; however autoclaved extracts had less anthocyanin content than filtered extracts.

H. sabdariffa extracts were effective against foodborne pathogens in a microbiological media and in two food systems, UHT milk and as an antimicrobial rinse on hot dogs. *Hibiscus* extracts have the potential to be a natural alternative to antimicrobials currently used in foods.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

Foodborne pathogens are a growing threat to public health and controlling their presence in foods is critical for maintaining the overall safety of the food supply. Pathogens have evolved mechanisms to overcome and resist harsh environments and the presence of antimicrobials. The discovery and use of effective natural antimicrobials is one way of combating these pathogens and overcoming resistance. In the United States, it is estimated that 9.4 million people will become sick from 31 major foodborne pathogens, requiring greater than 55,961 hospitalizations, and resulting in 1,351 deaths annually (Scallan and others 2011), with an economic burden of $51 billion (Scharff 2012). Pathogens such as methicillin-resistant *Staphylococcus aureus* and other multidrug-resistant pathogens are becoming more common in food products such as meats, and data suggests meat and poultry are often found to contain multidrug-resistant *S. aureus* (Waters and others 2011). Pathogens can become more virulent and more resistant to current treatments when they are exposed to sublethal doses of antibiotics.

Plants and their components have long been studied and examined for their antimicrobial activity. Plant phenolic compounds are secondary metabolites produced by plants in response to infection by pathogens or predators. Many of these compounds produced by plants have antimicrobial activity (Cowan 1999). *Hibiscus sabdariffa* is an annual, tropical or subtropical shrub species from the family Malvaceae and is native from Malaysia to India. The plant is commonly referred to as 'roselle' and grown commercially in numerous countries including Sudan, Mexico, India, and Thailand. Most species of *Hibiscus* are used as ornamentals, but the red calyces of *H. sabdariffa* are often used in the preparation of cold or hot beverages, which have been described as flavorful and tart. This beverage prepared from *Hibiscus* is frequently compared to cranberry due to this tart flavor (Morton 1987). Calyces have been shown to exhibit medicinal effects as

well as have antimicrobial properties. Numerous compounds have been identified from the calyces including anthocyanins, phenolic acids, organic acids, saponins, and alkaloids (Tsai and others 2002, Christian and Jackson 2009, Olaleye 2007) and may be contributing to its medicinal properties. Recently, Morales-Cabrera and others (2013) examined the antimicrobial activity of five varieties of *Hibiscus sabdariffa* against *Salmonella* Typhimurium and *S.* Choleraesuis. Aqueous methanol, and ethanol extracts were tested against *Salmonella* and it was determined that ethanol extracts were the most effective against *Salmonella* and pH (1.5-2.4) was a contributing factor to activity (Morales-Cabrera and others 2013). Additionally, drinking the tea has shown to lower blood pressure (McKay 2010, Faraji and Tarkhani 1999). Calyces also have known antimicrobial activity, but the research done using calyces in food systems against foodborne pathogens is limited. Antimicrobial activity of aqueous and ethanol extracts has been examined in ground beef and apple juice and found to be effective at inhibiting growth of pathogens such as *Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Listeria monocytogenes*, *Bacillus cereus*, and *Staphylococcus aureus* (Chao and Yin 2009). *Hibiscus sabdariffa* has also been shown to be effective as a wash on produce against *E. coli* O157:H7 and *Salmonella* (Jaroni and Ravishankar 2012). Extracts of *Hibiscus sabdariffa* calyces can provide health benefits due to the presence of antioxidants as well as serve as antimicrobials in foods. Identifying the specific compounds responsible for antimicrobial activity will provide novel antimicrobials that could be utilized in a variety of food products and serve as a natural alternative to current antimicrobials used in foods.

The objectives of this study were to examine the antimicrobial activity of *Hibiscus sabdariffa* aqueous calyx extracts in beverages (milk) and foods (hot dogs) against a wide-range of

foodborne pathogens (*Escherichia coli* O157:H7, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, and/or *Listeria monocytogenes*) and to compare the chemical composition and antimicrobial activity of 3 varieties of *H. sabdariffa* produced in the greenhouse to commercially acquired calyces.

References

Chao CY, Yin MC. 2009. Antibacterial effects of roselle calyx extracts and protocatechuic acid in ground beef and apple juice. Foodborne Path Dis 6: 201-6.

Christian KR, Jackson JC. 2009. Changes in total phenolic and monomeric anthocyanin composition and antioxidant activity of three varieties of sorrel (*Hibiscus sabdariffa*) during maturity. J Food Comp Anal 22:663-7.

Cowan MM. 1999. Plant products as antimicrobial agents. Clin Microbiol Rev 12:564-82.

Faraji MH, Tarkhani AHH. 1999. The effect of sour tea (*Hibiscus sabdariffa*) on essential hypertension. J Ethnopharmacol 65:231-6.

Jaroni D, Ravishankar S. 2012. Bactericidal effects of roselle (*Hibiscus sabdariffa*) against foodborne pathogens *in vitro* and on romaine lettuce and alfalfa sprouts. QAS 4:33-40.

McKay DL, Chen CYO, Saltzman E, Blumberg JB. 2010. *Hibiscus sabdariffa* L. tea (tisane) lowers blood pressure in prehypertensive and mildly hypertensive adults. J Nutr 140: 298-303.

Morales-Cabrera M, Hernandez-Morales J, Leyva-Ruelas G, Salinas-Moreno Y, Soto-Rojas L, Castro-Rosas J. 2013. Influence of variety and extraction solvent on antibacterial activity of roselle (*Hibiscus sabdariffa* L.) calyces. J Med Plants Res 7:2319-22.

Olaleye MT. 2007. Cytotoxicity and antibacterial activity of methanolic extract of *Hibiscus sabdariffa*. J Med Plants Res 1:9-13.

Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM. 2011. Foodborne illness acquired in the United States--major pathogens. Emerg Infect Dis 17:7-15.

Tsai PJ, McIntosh J, Pearce P, Camden B, Jordan BR. 2002. Anthocyanin and antioxidant capacity in roselle (*Hibiscus sabdariffa* L.) extract. Food Res Int 35: 351-6.

Chapter 2: Literature Review

2.1 Hibiscus sabdariffa

2.1.1 Cultivation and processing

Hibiscus sabdariffa (roselle) is an annual, tropical or subtrobical shrub native from Malaysia to India (Morton 1987). There are two main types of *Hibiscus sabdariffa*: var. *altissima* Wester cultivated mainly for its fiber and var. *sabdariffa* in which some cultivars give edible calyces (Morton 1987). The plant is commonly referred to as roselle and grown commercially in numerous countries including Sudan, Mexico, India, and Thailand. Most species of *Hibiscus* are used as ornamentals, but the red calyces of *H. sabdariffa* are often used in the preparation of cold or hot beverages.

Calyces are typically harvested 2-3 wk after flowering (Cisse and others 2009). Following flowering, the bloom wilts and falls off leaving the calyx (sepal) to swell and become bright red in color. The calyx surrounds a fruit containing seeds. The seeds are removed from the fruit and the calyx is dried naturally in the sun or artificially in ovens (Cisse and others 2009). Following drying, calyces are then packaged and distributed for purchase and use. Flowering can be extended by harvesting early and not allowing the fruits to mature (Morton 1987).

2.1.2 Uses of the calyces

The edible calyces are commonly used in the preparation of hot or cold beverages. The flavor of *Hibiscus* is frequently compared to cranberry due its tartness (Morton 1987). Calyces are typically boiled in water and sugar is often added to reduce this tartness. While the most common use for the calyces are for making a beverage, there are many other documented uses.

The calyces have been used to make other foods such as jams and jellies or added directly to salads. Additionally, roselle has been used as a coloring agent. The calyces are considered GRAS (generally recognized as safe) and are approved for use as a coloring agent in the U.S. by the Food and Drug Administration (21 CFR 172.510).

2.1.3 Constituents found in the calyces

The proximate composition of the calyces has been examined. Red calyces (based on 100 g dry weight) contain 6.4% protein, 79.25% carbohydrates, 5.13% fat, 2.7% crude fiber, and 6.52% ash (Nnam and Onyeke 2003).

The calyces of *Hibiscus sabdariffa* are rich in bioactive compounds. Organic acids are prevalent in the calyces resulting in the low pH of approximately 2-2.5. The major organic acids in the calyces are citric and malic acids while other acids such as tartaric have also been identified (Ali and others 2005).

The calyces are also rich in phenolic compounds. Some of the phenolic acids identified from *Hibiscus* calyces include gallic, chlorogenic, and protocatechuic acid (Ramirez-Rodrigues and others 2011). The total phenolic content observed varies depending on extraction method and/or *Hibiscus* variety. Phenolic content has been found to be 77.2 mg GAE (gallic acid equivalents)/g for an aqueous extract and 87.7 mg GAE/g for an ethanol extract (Al-Hashimi 2012). The phenolic content of an extraction of 1 g calyces in ethanol, which was evaporated, was found to be between 4.73-23.12 mg GAE/g depending on variety used (Christian and Jackson 2009). The

phenolic content of an extraction performed with hot water (1 g of calyx tea in 100 ml hot water) was found to be 13.3 mg GAE/g dry tea (Oboh and Rocha 2008). The anthocyanins, which are a type of flavonoid, are responsible for the deep red color of the calyces. The two major anthocyanins found are delphinidin-3-sambubioside and cyanidin-3-sambubioside (Du and Francis 1973). These compounds have been determined to be the major contributors of antioxidant activity (Tsai and others 2002). Other compounds present are gossypetin, quercetin, pectin (Hirunpanich and others 2005), saponins, cardiac glycosides, and alkaloids (Olaleye 2007). The levels of some of these compounds have been determined by HPLC. Ramirez-Rodrigues and others (2011) examined a dried calyx extract performed with cold water and determined the content present to be 8.19 mg/l for hibiscus acid derivates, 0.71 mg/l for hydroxybenzoic acids, 132.62 mg/l for caffeoylquinic acids, 29.74 mg/l for flavonols, and 128.94 mg/l for anthocyanins.

2.1.4 Medicinal properties

The medicinal properties of *Hibiscus sabdariffa* are numerous. In folk medicine, calyces are believed to be beneficial in reducing blood pressure. Several studies have confirmed this antihypertensive activity. Faraji and Tarkhani (1999) observed that blood pressure decreased 11.2 % for systolic and 10.7 % for diastolic in people after drinking the tea for 12 d. A similar study found that drinking *Hibiscus* tea three times daily demonstrated a reduction in blood pressure in pre-hypertensive and mildly hypertensive adults (McKay 2010). Capsules of the extract (500 mg capsules each containing 20.1 mg anthocyanins, 10.0 mg flavonoids, and 14.0 mg polyphenols) demonstrated a significant reduction in serum cholesterol in men and women (Lin and others 2007). Further, anthocyanins from *Hibiscus* were extracted and tested against

human promyelocytic leukemia cells (HL-60) to determine their effectiveness at causing apoptosis of these cells. The anthocyanins demonstrated to be inhibitory against the HL-60 cells and were determined to be possible chemopreventative agents (Chang and others 2005). Additionally, consuming 1.5 g of *Hibiscus* tea twice a day for 15 days showed a uricosuric effect, which helps with uric acid secretions and decreases the risk of renal stones (Prasongwatana and others 2008).

2.1.5 Antimicrobial studies

The antimicrobial properties of *Hibiscus* calyces have been studied and were demonstrated to be effective against a wide range of pathogens. Methanol extractions of the calyces have shown to have antimicrobial activity against *Staphylococcus aureus, Bacillus stearothermophilus, Micrococcus luteus, Serratia mascences, Clostridium sporogenes, Escherichia coli, Klebsiella pneumoniae, Bacillus cereus, and Pseudomonas* sp. (Olaleye 2007). The minimum inhibitory concentration (MIC) of the methanol extracts was found to be 0.30 ± 0.2 mg/ml for *S. aureus* and 1.30 ± 0.2 mg/ml for *E. coli* (Olaleye 2007). Crude extracts have also shown to have antibacterial activity against the acne-causing bacteria *Propionibacterium acnes* (MIC: 2.5 mg/ml, MBC: 5 mg/ml) and *Staphylococcus epidermidis* (MIC: 0.625 mg/ml, MBC: 5 mg/ml) (Chomnawang and others 2005). Aqueous extracts were also shown effective against methicillin-resistant *S. aureus, K. pneumoniae, Pseudomonas aeruginosa,* and *Acinetobacter baumannii* with the minimum inhibitory concentrations of 32 ± 8, 48 ± 2, 56 ± 8 and 48 ± 8 mg/l respectively (Liu and others 2005). Navarro Garcia and others (2006) found the MIC for aqueous *H. sabdariffa* extracts to be

between 0.5 and 8.0 mg/ml, the lowest for *S. aureus* and *Streptococcus faecalis* and the highest for *Candida albicans*.

Limited research has been conducted demonstrating this extracts' possible use as an antimicrobial in foods. Ethanol and aqueous extracts of *H. sabdariffa* (5 or 10 mg added to 100 g ground beef or 100 ml apple juice) showed inhibitory effects against *E. coli* O157:H7, *Salmonella* Typhimurium, *L. monocytogenes, B. cereus, and S. aureus* in ground beef and apple juice after 3 days of storage conditions with the ethanol extracts being more effective (Chao and Yin 2009). Recently, a study was performed which examined using aqueous extracts of *H. sabdariffa* (100 % v/v) as a wash on lettuce against *E. coli* O157:H7 and sprouts against *Salmonella enterica*. Bacterial populations of approximately 4 log CFU/g of *E. coli* O157:H7 and *Salmonella enterica* were destroyed after 24 hr (Jaroni and Ravishankar 2012).

The calyces have been shown to contain organic acids, which may be contributing to the activity. The undissociated form of the organic acid can enter the cell wall of bacteria. The lower the pH, the more undissociated form is present, which stresses the bacterial cell and can lead to death (Brul and Coote 1999). The pH of the *Hibiscus* extract is approximately 2-2.5. Low pH has shown to contribute to the antimicrobial activity of the extracts, and extracts with lower pH were found to be more effective against *Salmonella* (Morales-Cabrera and others 2013).

2.1.6 Toxicity

The oral toxicity of the calyx extracts is an important consideration when exploring their potential use as a food additive. Toxicity of *Hibiscus* has been examined, and it was determined

to be relatively non-toxic. Akindahunsi and Olaleye (2003) examined the effects of methanolic *Hibiscus sabdariffa* extracts and found that 150-180 mg/kg per day was virtually safe when given to rats, but higher doses had the potential to cause liver damage. When potentially consuming phenolic compounds, it is important not to exceed recommended levels, as these compounds have the potential to be dangerous to human health. A recent study performed with rats on the toxicity of aqueous extracts of *H. sabdariffa* calyces showed that acute (5000 mg/kg for 14 days) and chronic (50, 100, or 200 mg/kg for 270 days) toxicity was not significantly different from the control (Sireeratawong and others 2013).

2.2 Foodborne pathogens

2.2.1 *Escherichia coli* O157:H7

E. coli O157:H7 is a Gram-negative foodborne pathogen, wherein the 'O' refers to the somatic antigen and the H refers to the flagellar antigen. Disease symptoms can be observed with a relatively low infectious dose (Griffin and Tauxe 1991), as low as 10 CFU (Jay and others 2005), and can develop into hemolytic uremic syndrome (HUS) in children, elderly, or people with compromised immune systems. This pathogen has the ability to produce shiga-like toxins and adhere to intestinal mucosal cells (Griffin and Tauxe 1991). Due to its inability to ferment sorbitol rapidly, it is often isolated or identified by plating on sorbitol MacConkey agar and oberving for colorless colonies (Griffin and Tauxe 1991). Transmission of this organism is through the fecal/oral route, usually from contaminated food, water, or surfaces. Most outbreaks are the result of contaminated food, specifically of bovine origin (Griffin and Tauxe 1991); however, it has also been linked to outbreaks from other foods such as juices. Besser and others (1993) found *E. coli* O157:H7 in fresh pressed apple cider and determined its presence could be

reduced by washing and scrubbing the apples prior to processing. Currently, all juices are required to be pasteurized to achieve a 5 log reduction (21 CFR 120). *E. coli* O157:H7 is also acid tolerant and can adapt and survive at a lower pH when first exposed to an intermediate pH (around 5.0). Leyer and others (1995) observed that *E. coli* O157:H7 was more resistant to lactic acid if first exposed to acidic condtions and also exhibited increased survival in foods such as dry salami and sausage.

2.2.2 *Listeria monocytogenes*

L. monocytogenes is a Gram-positive non-spore forming rod. This pathogen is a concern for the food industry due to its ubiquitous nature, its ability to survive and grow at lower temperatures (1 to 45 °C), and its ability to form biofilms, making it difficult to eradicate (Jay and others 2005). *Listeria* has an optimal pH of 6-8 but has been found to be able to grow in the pH range of 4.1-9.6 (Jay and others 2005). The habitat for this microorganism is numerous and can be found in soil, vegetables, water, healthy animals, and many other common environments. This pathogen causes the disease, listeriosis, which can cause symptoms such as fever, muscle aches, and diarrhea (CDC 2013). It is able to cross the placenta causing concern for pregnant women, often resulting in spontaneous abortion. A low infectious dose of < 1000 CFU can cause illness (Jay and others 2005), but this dose is typically for those at higher risk. Healthy individuals are usually asymptomatic after consuming *Listeria* contaminated foods. A study conducted by the CDC found that people 65 and older were four times more likely and pregnant women were found to be ten times more likely to be affected by *Listeria* (CDC 2013). *Listeria monocytogenes* is estimated to cause 1,455 hospitalizations annually with 255 deaths in the U.S. (Scallan and others 2011).

Foods that are at highest risk for being contaminated with *Listeria* are ready-to-eat (RTE) and stored at refrigeration temperatures for long periods of time. *Listeria* has been associated with outbreaks from numerous food products including meats, milk, cheeses, and produce. An outbreak occurred from contaminated chocolate milk in 1994 and the illnesses were determined to be caused by *L. monocytogenes* serotype 1/2b. Symptoms included diarrhea and fever and patients had higher levels of antibodies in response to listeriolysin O (Dalton and others 1997). Another outbreak occurred in 1998 from contaminated hot dogs (Bil Mar Foods) resulting in 6 deaths and 2 spontaneous abortions (CDC 1998). *Listeria* has also been recently linked to cantaloupes. An outbreak of listeriosis occurred in 2011 that was linked to cantaloupes from Jensen Farms in Colorado, which resulted in 147 illnesses and 33 deaths (CDC 2012).

L. monocytogenes has numerous virulent factors. It can be an intracellular pathogen and cause invasive illness in humans. Listeriolysin O, a hemolysin, is a known virulence factor that is necessary for intracellular growth and survival (Cossart and others 1989). This allows *Listeria* to evade the immune response. *L. monocytogenes* also has a surface protein, ActA, which needs the host cell Arp2/3 complex to begin actin polymerization and this allows the bacterium to propel itself (Welch and others 1997).

2.2.3 Staphylococci

S. aureus is a Gram-positive facultative anaerobic cocci that produces enterotoxins, which can cause staphylococcal gastroenteritis when consumed (Jay and others 2005). This intoxication leads to symptoms such as vomiting, cramps, and diarrhea. The illness usually subsides on its own within a couple of days. People usually get sick from foods that were made by hand and

then stored improperly, allowing the toxins to be produced and accumulate. Humans are a natural reservoir and asymptomatic colonization is typical (Chambers 2001). Thirty to 50% of adults are colonized and transmission of nosocomial infections are usually the result of exposure to others who are colonized (Lowy 1998). *S. aureus* is an adaptive pathogen in that it is able to withstand a variety of intrinsic and extrinsic factors of foods. Growth occurs in the temperature range of 7 to 47.8 °C while enterotoxins are produced between 10 and 46 °C (Jay and others 2005). Growth of the enterotoxins can be very rapid depending on the conditions and strain and also occurs during all phases of bacterial growth (Jay and others 2005). *S. aureus* can grow in foods with pH values ranging from 4 to 9.8 (Medvedova and Valik 2012), has been shown to withstand salt (7-10%), and can grow in foods with water activity as low as 0.86 (Jay and others 2005).

Biofilm formation is also common among *S. aureus* species. Rode and others (2007) found that *S. aureus* strains had increased biofilm formation at suboptimal temperatures as well as in the presence of both sodium chloride and glucose. These conditions are common in food a processing plant, which makes contamination by this pathogen a concern.

Resistance to antibiotics is also a concern when it comes to many bacteria, especially *S. aureus*, which has evolved and developed resistance to several antibiotics. Methicillin-resistant *S. aureus* (MRSA) infections are becoming more common and seen a lot more in the community rather than just in hospitals. Surveillance performed between July 2004 through December 2005 found 8987 infections and 58.4% of those were community-onset (Klevens and others 2007). MRSA contains the *mecA* gene, which encodes a different penicillin binding protein (PBP2a) that prevents β-lactam antibiotics from binding (Malachowa and DeLeo 2010). *mecA* is also tightly

regulated by the genes *blaI* and *blaR1* (Hackbarth and others 1993). With MRSA being found in meat products more often, there is a concern that consumers may contract the pathogen from improper handling of fresh meat or through ready-to-eat products where contamination can occur post processing. If MRSA were present on meat and the toxins were able to form, cooking the meat may not destroy the toxins. Heating for 11 min at 250 °F was required to destroy enterotoxin type A (Jay and others 2005). Even though most outbreaks of *S. aureus* are the result of contamination through improper hygiene and handling in restaurant or home settings, MRSA is a developing concern for the food industry. If MRSA were to get into meat at any point of processing, it could be spread to other areas of food production. MRSA, along with other nosocomial pathogens, have the ability to survive on surfaces for months (Kramer and others 2006), which could be especially problematic if surfaces were not sanitized properly. MRSA has been implicated in various retail meats. One particular strain, ST398, is often associated with animals in food production and has spread to humans (Kluytmans 2010). Humans colonized with ST398 were more likely to be associated with working on pig farms (Kluytmans 2010). Kluytmans (2010) highlighted the risks involved when MRSA is found in meat products with one major risk being the colonization of humans after handling contaminated meat.

References

Akindahunsi AA, Olaleye MT. 2003. Toxicological investigation of aqueous-methanolic extract of the calyces of *Hibiscus sabdariffa* L. J Ethnopharmacol 89:161-4.

Al-Hashimi AG. 2012. Antioxidant and antibacterial activities of *Hibiscus sabdariffa* L. extracts. African J Food Sci 6:506-11.

Ali BH, Al Wabel N, Blunden G. 2005. Phytochemical, pharmacological, and toxicological aspects of *Hibiscus sabdarrifa* L.: a review. Phytother Res 19:369-75.

Besser RE, Lett SM, Weber JT, Doyle MP, Barrett TJ, Wells JG, Griffin PM. 1993. An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh-pressed apple cider. JAMA 269:2217-20.

Brul S, Coote P. 1999. Preservative agents in foods: mode of action and microbial resistance mechanisms. Int J Food Microbiol 50:1-17.

CDC. 1999. Update: multistate outbreak of listeriosis—United States, 1998-1999. MMWR Weekly 47:1117-8.

CDC. 2012. Multistate outbreak of listeriosis linked to whole cantaloupes from Jensen Farms, Colorado. Available at: http://www.cdc.gov/listeria/outbreaks/cantaloupes-jensen-farms/. Accessed: October 15, 2013.

CDC. 2013. *Listeria* (listeriosis). Available at: http://www.cdc.gov/listeria/. Accessed: September 20, 2013.

Chang YC, Huang HP, Hsu JD, Yang SF, Wang CJ. 2005. *Hibiscus* anthocyanins rich extract-induced apoptotic cell death in human promyelocytic leukemia cells. Toxicol Appl Pharmacol 205: 201-12.

Chao CY, Yin MC. 2009. Antibacterial effects of roselle calyx extracts and protocatechuic acid in ground beef and apple juice. Foodborne Path Dis 6: 201-6.

Chomnawang MT, Surassmo S, Nukoolkarn VS, Gritsanapan W. 2005. Antimicrobial effects of Thai medicinal plants against acne-inducing bacteria. J Ethnopharacol 101:330-3.

Christian KR, Jackson JC. 2009. Changes in total phenolic and monomeric anthocyanin composition and antioxidant activity of three varieties of sorrel (*Hibiscus sabdariffa*) during maturity. J Food Comp Anal 22: 663-7.

Cisse M, Dornier M, Sakho M, MarDiop C, Reynes M, Sock O. 2009. Bissap (*Hibiscus sabdariffa* L.) production in Senegal. Fruits 64: 111-24.

Cossart P, Vicente MF, Mengaud J, Baquero F, Perez-Diaz JC, Berche P. 1989. Listeriolysin O is essential for virulence of *Listeria monocytogenes*: direct evidence obtained by gene complementation. Infect Immun 57:3629-36.

Dalton CB, Austin CC, Sobel J, Hayes PS, Bibb WF, Graves LM, Swaminathan B, Proctor ME, Griffin PM. 1997. An outbreak of gastroenteritis and fever due to *Listeria monocytogenes* in milk. N Engl J Med 336:100-6.

Du CT, Francis FJ. 1973. Anthocyanins of roselle (*Hibiscus sabdariffa,* L.) J Food Sci 38:810-2.

Faraji MH, Tarkhani AHH. 1999. The effect of sour tea (*Hibiscus sabdariffa*) on essential hypertension. J Ethnopharmacol 65:231-6.

Griffin PM, Tauxe RV. 1991. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli,* and the associated hemolytic uremic syndrome. Epidemiol Rev13:60-98.

Hackbarth CJ, Chambers HF. 1993. *blaI* and *blaR1* regulate beta-lactamase and PBP 2a production in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 37:1144-9.

Jaroni D, Ravishankar S. 2012. Bactericidal effects of roselle (*Hibiscus sabdariffa*) against foodborne pathogens *in vitro* and on romaine lettuce and alfalfa sprouts. QAS 4:33-40.

Jay JM, Loessner MJ, Golden DA. 2005. Modern food microbiology. 7th ed. New York: Springer. 790 p.

Leyer GJ, Wang LL, Johnson EA. 1995. Acid adaptation of *Escherichia coli* O157:H7 increases survival in acidic foods. Appl Environ Microbiol 61:3752-5.

Lin TL, Lin HH, Chen CC, Lin MC, Chou MC, Wang CJ. 2007. *Hibiscus sabdariffa* extract reduces serum cholesterol in men and women. Nutr Res 27:140-5.

Liu KS, Tsao SM, Yin MC. 2005. *In vitro* antibacterial activity of roselle calyx and protocatechuic acid. Phytother Res 19:942-5.

Malachowa N, DeLeo FR. 2010. Mobile genetic elements of *Staphylococcus aureus*. Cell Mol Life Sci 67:3057-71.

McKay DL, Chen CYO, Saltzman E, Blumberg JB. 2010. *Hibiscus sabdariffa* L. tea (tisane) lowers blood pressure in prehypertensive and mildly hypertensive adults. J Nutr 140: 298-303.

Morton J. 1987. Roselle. p. 281-286. In: Fruits of warm climates. Miami, FL. http://www.hort.purdue.edu/newcrop/Morton/roselle.html.

Navarro Garcia VM, Rojas G, Zepeda LG, Aviles M, Fuentes M, Herrera A, Jimenez E. 2006. Antifungal and antibacterial activity of four selected mexican medicinal plants. Plarm Biol 44:297-300.

Nnam NM, Onyeke NG. 2003. Chemical composition of two varieties of sorrel (*Hibiscus sabdariffa* L.), calyces and the drinks made from them. Plant Foods Human Nutr 58:1-7.

Olaleye MT. 2007. Cytotoxicity and antibacterial activity of methanolic extract of *Hibiscus sabdariffa*. J Med Plants Res 1: 9-13.

Obah G, Rocha BT. 2008. Antioxidant and neuroprotective properties of sour tea (*Hibiscus sabdariffa,* calyx) and green tea (*Camellia sinensis*) on some pro-oxidant-induced lipid peroxidation in brain *in vitro*. Food Biophys 3: 382-9.

Prasongwatana V, Woottisin S, Sriboonlue P, Kukongviriyapan V. 2008. Uricosuric effect of roselle (*Hibiscus sabdariffa*) in normal and renal-stone former patients. J Ethnopharmacol 117:491-5.

Ramierez-Rodrigues MM, Plaza ML, Azeredo A, Balaban MO, Marshall MR. 2011. Physiochemical and phytochemical properties of cold and hot water extraction from *Hibiscus sabdariffa*. J Food Sci 76:C428-35.

Rode TM, Langsrud S, Holck A, Moretro T. 2007. Different patterns of biofilm formation in *Staphylococcus aureus* under food-related stress conditions. Int J Food Micrbiol 116:372-83.

Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM. 2011. Foodborne illness acquired in the United States--major pathogens. Emerg Infect Dis 17:7-15.

Sireeratawong S, Itharat A, Khonsung P, Lertprasertsuke N, Jaijoy K. 2013. Toxicity studies of the water extract from the calyces of *Hibiscus sabdariffa* L. in rats. African J Trad Complement Alt Med 10:122-7.

Tsai PJ, McIntosh J, Pearce P, Camden B, Jordan BR. 2002. Anthocyanin and antioxidant capacity in roselle (*Hibiscus sabdariffa* L.) extract. Food Res Int 35: 351-6.

Weigel LM, Clewell DB, Gill SR, Clark NC, McDougal LK, Flannagan SE, Kolonay JF, Shetty J, Killgore GE, Tenover FC. 2003. Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*. Science 302:1569-71.

Welch MD, Iwamatsu A, Mitchison TJ. 1997. Actin polymerization is induced by Arp2/3 protein complex at the surface of *Listeria monocytogenes*. Nature 385:265-9.

Chapter 3: Antimicrobial activity of *Hibiscus sabdariffa* aqueous extracts against *Escherichia coli* O157:H7 and *Staphylococcus aureus* in a microbiological medium and milk of varying fat concentrations

Adapted from:

Kristen L. Higginbotham, Kellie P. Burris, Svetlana Zivanovic, P. Michael Davidson, C. Neal Stewart, Jr. Antimicrobial activity of *Hibiscus sabdariffa* aqueous extracts against *Escherichia coli* O157:H7 and *Staphylococcus aureus* in a microbiological medium and milk of varying fat concentrations. Journal of Food Protection. In Press.

Kristen Higginbotham wrote the majority of the manuscript, executed the experiments and analyzed the data.

3.1 Abstract

Hibiscus sabdariffa L. calyces are widely used in the preparation of beverages. The calyces contain compounds that exhibit antimicrobial activity, yet little research has been conducted on their possible use in food systems as antimicrobials. Aqueous extracts prepared from the brand 'Mi Costenita' were sterilized by membrane filtration (0.22 µm) or autoclaving (121°C, 30 min) and tested for antimicrobial activity against the foodborne pathogens, *Escherichia coli* O157:H7 strains ATCC 43894 and 'Cider' and *Staphylococcus aureus* strains SA113 and ATCC 27708 in a microbiological medium and ultrahigh temperature-processed (UHT) milk with various fat percentages. Extracts heated by autoclaving exhibited greater activity compared to filtered extracts in a microbiological medium. Against *E. coli*, 20 mg/ml filtered extract was not different than the control; whereas autoclaved extracts reduced viable cells ca. 3-4 log CFU/ml. At 60 mg/ml, both extracts inactivated cells after 24 h. There were reduced populations of both strains of *S. aureus* (ca. 2.7 and 3 log CFU/ml, respectively) after 24 h of incubation in 40 mg/ml filtered extracts. When grown in autoclaved extracts at 40 mg/ml, both strains of *S. aureus* were inactivated after 9 h. Autoclaved extracts had decreased anthocyanin content (2.63 mg/l) compared with filtered extracts (14.27 mg/l), whereas the phenolic content (48.7 and 53.8 mg/g) remained similar for both treatments. Autoclaved extracts were then tested for activity in milk at various fat concentrations (skim [< 0.5%], 1%, 2%, and whole [>3.25%]) against a 1:1 mixture of both strains of *E. coli* O157:H7 and a 1:1 mixture of both strains of *S. aureus*. Extracts at 40 mg/ml inactivated *S. aureus* after 168 h in skim and whole milk and *E. coli* was inactivated after 96 h in 60 mg/ml extract in all fat levels. These findings show the potential use of *Hibiscus* extracts to prevent the growth of pathogens in foods and beverages.

Keywords: *Hibiscus sabdariffa,* antimicrobial activity, UHT milk, *E. coli* O157:H7, *S. aureus*

3.2 Introduction

According to the Centers for Disease Control and Prevention, an estimated 48 million people are sickened by foodborne diseases each year in the U.S. (CDC 2011). Shiga-toxin-producing *Escherichia coli* (STEC) O157:H7 causes an estimated 63,000 illnesses annually resulting in 20 deaths (Scharff 2012). *Staphylococcus aureus* is estimated to cause 240,000 foodborne related illnesses each year with greater than 1000 hospitalizations and 6 deaths (Scharff 2012). *S. aureus* is often transmitted from human contamination and produces enterotoxins, which can cause gastrointestinal illness when consumed (Jay and others 2005). There are 13 known staphylococcal enterotoxins and the form(s) of enterotoxin encoded depend on the isolate (Jay and others 2005). Foodborne illnesses are also costly, resulting in expenses of approximately $51 billion each year in the United States (Scharff 2012). It is a worthy goal to decrease the number of illnesses from foodborne pathogens.

Novel antimicrobials from natural sources are new tools with the potential to help ensure a safe food supply while decreasing the use of synthetic antimicrobials. *Hibiscus sabdariffa* L. (family Malvaceae) is an annual, tropical or subtropical shrub that is native from Malaysia to India. *Hibiscus* is grown in many countries including Sudan, Mexico, India, and Thailand. While many species of *Hibiscus* are used as ornamentals, the red calyces of *H. sabdariffa* are used in the preparation of a flavorful and tart cold or hot beverage. These calyces have been shown to contain numerous bioactive compounds. The majority of compounds found within the calyces that exhibit antimicrobial activity are the polyphenolic compounds, some of which have also been shown to demonstrate antioxidant activity (Haslam 1998; Tsai and others 2002). One group of polyphenolic compounds present in *Hibiscus* calyx extract is the flavonoids, which includes

the plant pigments, anthocyanins. The two anthocyanins, present in the highest amount, that have been identified in *Hibiscus*, delphinidin-3-sambubiside and cyanidin-3-sambubioside (Du and Francis 1973), are responsible for the deep red pigment of the calyces and were also found to be the major contributors to antioxidant activity (Tsai and others 2002). Other compounds found in the calyces include phenolic acids such as gallic and protocatechuic acid (Ramirez-Rodrigues and others 2011).

Hibiscus extracts have been shown to have a wide range of antimicrobial activity against bacteria. Methanol extractions of the calyces have shown to have antimicrobial activity against *S. aureus, Bacillus stearothermophilus, Micrococcus luteus, Serratia marcescens, Clostridium sporogenes, E. coli, Klebsiella pneumoniae, Bacillus cereus, and Pseudomonas* sp. at concentrations of 0.30 ± 0.2 to 1.30 ± 0.2 mg/ml (Olaleye 2007). Navarro Garcia and others (2006) found the MIC for aqueous, calyx (*Hibiscus sabdariffa*) extracts was 0.5 and 1.0 mg/ml for *S. aureus* ATCC 6358 and *E. coli* ATCC 8937. However, there is limited research regarding the extracts' possible use as antimicrobials in foods and beverages. Ethanol and aqueous extracts of *H. sabdariffa* (5 or 10 mg added to 100 g ground beef or 100 ml apple juice) showed dose dependent inhibitory effects against *E. coli* O157:H7, *Salmonella* Typhimurium, *Listeria monocytogenes, B. cereus, and S. aureus* after 3 days of storage conditions, with ethanol extracts demonstrating greater antimicrobial activity (Chao and Yin 2009). Recently, a study was performed that examined the use of aqueous extracts of *H. sabdariffa* (100% v/v) as a wash on lettuce against *E. coli* O157:H7 and sprouts against *Salmonella enterica*. Bacterial populations of approximately 4 log CFU/g of *E. coli* O157:H7 and *S. enterica* were eliminated after 24 h (Jaroni and Ravishankar 2012).

The objective of this study was to determine the antimicrobial effectiveness of aqueous *Hibiscus* extracts against *E. coli* O157:H7 and *S. aureus* in milk as a model food system, and explore the chemical composition of both filtered and autoclaved extracts.

3.3 Materials and Methods

3.3.1 Preparation of extract

Aqueous extracts from the dried calyces (*Hibiscus sabdariffa)* of the brand 'Mi Costenita', purchased from a local international market (Knoxville, TN), were prepared according to published methods (Burris and others 2012) with modifications. Dried calyces were finely ground (<1 mm) with a blender (Oster, Boca Raton, FL). Extracts were obtained by adding sterile water at a ratio of 3.6 ml to 1 g ground tissue and allowed to stand for 2 h at 4°C in the dark with occasional mixing to maximize extraction. The mixture was then centrifuged at 5000 x g for 30 min to remove large particles. Aqueous extracts were then dialyzed (3500 MWCO; SnakeSkin® Pleated Dialysis Tubing, Pierce Biotechnology, Rockford, IL) for 36 h against deionized water with three water changes at 4 °C in the dark to remove low molecular weight compounds. Resulting extracts were centrifuged at 5000 x g for 30 min to remove sediment and then frozen at -80°C. Frozen extracts were lyophilized using LabConco FreeZone 12 Liter Freeze Dry System (Kansas City, MO) and stored in a sealed container at room temperature.

3.3.2 Preparation of cultures

E. coli O157:H7 strains ATCC 43894 and 'Cider' were stock cultures obtained from the Department of Food Science and Technology at the University of Tennessee, Knoxville and *S. aureus* strains SA113 and ATCC 27708 were obtained from the Center for Environmental Biotechnology at the University of Tennessee, Knoxville (courtesy of Dr. Steven Ripp). Cultures were grown in tryptic soy broth (TSB; Becton, Dickinson and Co., Sparks, MD) and stored in glycerol at -20°C. Working cultures were obtained by inoculating 50 ml TSB with 200 µL stock cultures and then incubating them for 24 h at 35-37 °C. *S. aureus* strain SA113 was subcultured once due to slower growth. After incubation, cultures were diluted to ca. 5.0-6.0 log CFU/ml and tested for antimicrobial activity.

3.3.3 Time-kill assays

Processed lyophilized extracts were re-suspended in 10 ml sterile water and filtered through 0.22 µm Express PES Membrane (Millipore, Billerica, MA) or autoclaved at 121°C for 30 min to sterilize. Antimicrobial activity of filtered and autoclaved samples was compared in a microbiological medium against each strain of *E. coli* O157:H7 (final concentrations of 20, 40, and 60 mg/ml extract) and *S. aureus* (final concentrations of 2.5, 20, and 40 mg/ml extract). Autoclaved samples at the final concentrations of 60 and 40 mg/ml were then tested in ultrahigh temperature-processed (UHT) milk against a 1:1 mixture of *E. coli* O157:H7 and a 1:1 mixture of *S. aureus* strains, respectively. Re-suspended extracts were mixed with 2.5 ml bacteria harvested at late logarithmic phase and diluted to ca. 5.0-6.0 log CFU/ml. Bacteria and extracts were incubated in 12.5 ml TSB at 35-37 °C or UHT milk at varying fat concentrations (skim milk [<0.05% fat] Hershey's, Hershey, PA and 1% fat, 2% fat, whole milk [>3.25% fat]

Parmalat, Parma, Italy) at room temperature. At regular intervals (0, 3, 6, and 24 h (*E. coli*) or 0, 3, 6, 9, 24 h (*S. aureus*) for TSB and 0, 6, 24, 48, 96, and 168 h for UHT milk) a bacterial suspension sample was collected, serially diluted in 0.1% peptone, plated in duplicate using tryptic soy agar (TSA; Becton, Dickinson and Co., Sparks, MD), and incubated for 24 h at 35-37 °C. The next day CFU were enumerated. Controls were prepared in a similar way using sterile water. The pH of the milk was decreased to 3.7 after the addition of the *Hibiscus* extracts, thus the milk used for the controls was adjusted with 1 N HCl so that all milk samples had a pH of 3.7.

3.3.4 Anthocyanin and phenolic content

The pH differential method (Lee and others 2005) was used to determine the anthocyanin content present in the extracts (either filtered through a 0.22 µm membrane or autoclaved at 121 °C for 30 min) at the concentration of 1 mg/ml. Absorbance was read at 520 nm and 700 nm at pH 1.0 and 4.5 with a dilution factor of 3 expressed in mg cyanidin-3-glucoside equivalents/l (MW = 449.2, ε = 26,900). To determine the amount of polymerized color, extracts were bleached with a bisulfite solution and the absorbance was measured at 420, 520, and 700 nm (Giusti and Wrolstad 2001). Percent polymeric color = (polymeric color/color density) x 100, where the equations for polymeric color and color density = $[(A_{420nm} - A_{700nm}) + (A_{520nm} - A_{700nm})]$ x dilution factor. The color density is based on water-diluted extract, whereas the polymeric color is based on the extract diluted with the bisulfite solution (Guisti and Wrolstad 2001). Phenolic content was determined using Folin-Ciocalteu's phenol reagent (Montreau 1973). Rehydrated extracts (1 mg/ml) were either filtered through 0.22 µm or autoclaved. Extracts were additionally filtered through Whatman No. 4 after cooling. The phenolic content was quantified using gallic

acid (GA) as the standard, and expressed in mg gallic acid equivalents (GAE)/g dry calyx extract after the absorbance was read at 725 nm.

3.3.5 Statistical analysis

Results for the anthocyanin and phenolic content were tabulated as the mean value of three replications ± standard error. For the time kill assays, the experiments were repeated twice and duplicate plating was also used. Statistical analysis was performed in a completely randomized design by analysis of variance (ANOVA) to test for significance. The general linear model was used (SAS 9.3, SAS Institute, Cary, NC). Least significant differences (LSD) were used to identify significant differences (at the 0.05 probability level) among treatment mean values. Error bars presented represent 95 % confidence intervals using LSD.

3.4 Results

Antimicrobial activity of filtered and autoclaved extracts in a microbiological medium. Processed *Hibiscus* extracts demonstrated antimicrobial activity against *E. coli* O157:H7 and *S. aureus* both in a microbiological medium and UHT milk. In TSB, the extracts lowered the pH to ca. 3.5. Filtered extracts at 20 mg/ml caused an increased lag phase of both strains of *E. coli* O157:H7 of 3 h but the final levels of growth in extracts at 20 mg/ml reached ca. 7.6-8.6 log CFU/ml compared to ca. 8.7-9.9 log CFU/ml for the control (0 mg/ml) after 24 h in TSB at 35-37 °C for strains ATCC 43894 and 'Cider' respectively (Fig. 1A,B). Filtered extracts at 40 mg/ml resulted in growth inhibition of both strains; however, inhibition of ATCC 43894 was essentially bacteriostatic at 24 h while the same concentration reduced strain 'Cider' by ca.4.5 log CFU/ml. Both strains of *E. coli* O157:H7 were inactivated to the level of detection after 24 h

in 60 mg/ml filtered extracts. Autoclaved extracts showed enhanced activity at similar concentrations to filtered extracts. At 20 mg/ml, autoclaved extracts were more effective than filtered extracts against both strains of *E. coli* O157:H7. The population of the strain 'Cider' was reduced by ca. 4 and 5 log CFU/ml at 20 mg/ml and ATCC 43894 by ca. 3 log CFU/ml (Fig. 1C,D). With 40 mg/ml of autoclaved extracts, inhibition of 'Cider' was similar to that observed with the filtered extract but against ATCC 43894 inhibition was enhanced to at least a 6 log reduction. Autoclaved extracts at 60 mg/ml were similarly effective at inactivating both strains of *E. coli* O157:H7 after 24 h except that inactivation of strain 'Cider' occurred after 6 h.

Lower apparent concentrations of filtered and autoclaved extracts of *H. sabdariffa* were required to inactivate *S. aureus* compared with *E. coli.* (Fig. 2). Filtered extracts at 2.5 mg/ml and 20 mg/ml caused an increase in the lag phase of both strains of *S. aureus* but only 20 mg/ml demonstrated bacteriostatic activity after 24 h. Reductions of ca. 2.7 and 3 log CFU/ml of *S. aureus* strains ATCC 27708 and SA 113, respectively, were seen in filtered extracts at 40 mg/ml after 24 h (Fig. 2A,B). In contrast, populations of SA113 and ATCC 27708 were reduced ca. 5.4 and 1.7 log CFU/ml, respectively, when exposed to autoclaved extracts at 2.5 mg/ml after 24 h. Reductions of ca. 0.7 and 1.9 log CFU/ml of SA113 and ATCC 27708, respectively, were observed in 20 mg/ml. It is not clear why the 2.5 mg/ml extract was more effective than the 20 mg/ml extract against the strains, particularly with SA113. Inactivation to undetectable levels of both strains of *S. aureus* was observed after 9 h at 35-37 °C when grown in autoclaved extracts at 40 mg/ml.

Antimicrobial activity of autoclaved extracts in UHT milk. For each of the UHT milk treatments, no growth was observed in any of the uninoculated controls incubated at room temperature after 1 wk (data not shown). When grown at pH 3.7 and with the appropriate pH

controls, *E. coli* and *S. aureus* increased their population size by ca. 2.0-3.0 log CFU/ml by hour 168. Some curdling of the milk was observed after pH was lowered. Autoclaved extracts (60 mg/ml) were shown to be effective in milk at all levels of fat against *E. coli* O157:H7 (Fig. 3A). Inactivation to undetectable levels (ca. 5.5 log CFU/ml reduction) of *E. coli* O157:H7 was observed after 48 h in skim milk and 96 h in 1%, 2%, and whole milk with treatment of 60 mg/ml extracts. For *S. aureus*, 40 mg/ml extracts caused an initial bacteriostasis, but after 168 h the populations were reduced by ca. 5.5 log CFU/ml in skim and whole milk and by ca. 2.6 log CFU/ml in 1% and 2% UHT milk.

Compounds present in the extracts. Yield of lyophilized extract taken from 100 g of whole dried calyx ranged from 1.78 to 2.14 g. The phenolic content ranged from 48.7 to 53.8 mg GAE/g, while the monomeric anthocyanin content ranged from 2.63 to 14.27 mg cyanidin-3-glucoside equivalent/l. For the crude extract and phenolic amounts the lower value was from the filtered treatment, whereas the lower value for anthocyanins came from the autoclaved treatment (Table 1). The polymerized color of the extracts was 52% in the filtered treatment and 70% for the autoclaved treatment (Table 1). Apparently, autoclaving did not affect total phenolic content; however, it caused a significant degradation of anthocyanins.

3.5 Discussion

In this study, we examined the effects of autoclaving *Hibiscus* extracts on their antimicrobial activity, phenolic and anthocyanin content, and determined their use to prevent the growth of pathogens in the model food system, milk. Numerous compounds from *H. sabdariffa* have been identified, many of which are known to have antimicrobial activity, although it is not clear what specific compounds are responsible or if there might be a synergistic effect among all or some of

the compounds present. Phenolic compounds are known to exhibit antimicrobial activity. Calyx extracts contain many phenolic compounds such as hydroxybenzoic acids and caffeolyquinic acids (Ramirez-Rodrigues and others 2011). The protocatechuic acid content has been examined in both freeze dried aqueous and ethanol extracts of calyces and was determined to be 2.8 ± 0.7 and 11.9 ± 1.2 mg/g freeze-dried calyx extract respectively, and antibacterial activity of protocatechuic acid was also not affected by heat from 25 to 100 °C (Chao and Yin 2009). Aqueous calyx extracts were also shown effective against methicillin-resistant *S. aureus, K. pneumoniae, Pseudomonas aeruginosa,* and *Acinetobacter baumannii* with the MIC of 32 ± 8, 48 ± 2, 56 ± 8 and 48 ± 8 mg/l, respectively (Liu and others 2005). Flavanoids present could be forming complexes with the bacterial cell wall (Cowan 1999). The flavonoid, gossypetin, which is present in the calyces, has also been shown to exhibit antibacterial activity against both Gram-negative and Gram-positive pathogens (Mounnissamy and others 2002).

Autoclaved (121°C, 30 min) extracts were more effective against both the *E. coli* O157:H7 and *S. aureus* compared to the filtered extracts in a microbiological medium. Autoclaving may have produced smaller or additional compounds in the extracts which were more effective against these pathogens. Liu and others (2005) examined the effect of heating *H. sabdariffa* at 60 and 100°C for 60 min and found that the antimicrobial activity of the *Hibiscus* extract was not affected. However, Chao and Yin (2009) examined the effect of heat on aqueous extracts of *H. sabdariffa* and determined antimicrobial activity to be significantly reduced after heating the extract to 75 and 100°C against *S. aureus, E. coli* O157:H7, *L. monocytogenes, B. cereus,* and *Salmonella* Typhimurium, but activity of the ethanol extracts were not affected by temperatures of 50 and 75°C. Elsayed and Elshafei (2011) also examined boiling and autoclaving calyx

extracts and found the MIC was not significantly affected against *Bacillus mycoides, E. coli,* and *C. albicans,* compared to room temperature extracts. In the present study, autoclaved extracts exhibited enhanced activity compared to filtered extracts. The major difference in the present study was that extracts were only autoclaved following the entire extraction process while other studies heated the extracts prior to concentrating them.

Differences in phenolic and anthocyanin content were examined in both filtered and autoclaved extracts, given that calyces of *H. sabdariffa* are rich in both anthocyanins and phenolic compounds. Anthocyanins are not heat stable. Ramirez-Rodrigues and others (2012) also found that anthocyanins in *Hibiscus* beverages degrade over time during storage, more so in heat treated beverages than non-heat treated. In the present study, the anthocyanin content of the filtered extract (14.27 mg cyanidin-3-glucoside equivalent/l) is lower than that shown in other published studies. Ramirez-Rodrigues and others (2011) used dried calyces and a cold (25 °C) aqueous extraction (ratio 1:40) and determined the anthocyanin content to be 128.94 mg delphinidin-3-glucoside equivalent/l. Cisse and others (2009) examined 4 varieties of calyx extracts made using water at a ratio of 1:10. The varieties used were Guatemala, Koor, Vimto, and Thai, which had anthocyanin contents of 315, 250, 718, and 306 mg cyanidin-3-glucoside equivalent/l respectively. In the present study, the polymeric color of the autoclaved extract, or the complexes the anthocyanins form with other compounds such as tannins (Giusti and Wrolstad 2001), was shown to have approximately 20% more polymerization than the filtered extract. The phenolic content in the filtered and autoclaved extracts (48.7 and 53.8 mg GAE/g) is somewhat higher than data found in the literature. In this study, the extract was dialyzed and lyophilized, which could have concentrated the phenolic compounds. The phenolic content of an

ethanol extraction of traditional red calyces was found to be 16 mg GAE/g, which was performed using 1 g of fresh freeze dried calyces extracted with ethanol that was later evaporated (Christian and Jackson 2009). The phenolic content of an extraction done with hot water (1 g of calyx tea in 100 ml hot water) was found to be 13.3 mg GAE/g dry tea (Oboh and Rocha 2008). The differences observed may be the result of the extraction process. Similarly, variation in content has been observed in other plants depending on the extraction method. Oboh and Rocha (2008) extracted 1 g of green tea in 100 ml hot water and determined the phenolic content to be 24.5 mg GAE/g dry tea. Rusak and others (2008) examined various extraction methods and determined the phenolic content in green and white teas. Green and white teas (2 g), either bagged or loose, were extracted in 200 ml of water, water with lemon juice, or ethanol at 10, 40, and 70 % for times of 5, 15, and 30 min. They found that aqueous ethanol (40 %) for 30 min was the best to extract both teas in bagged form and white tea contained around 2100 mg GAE/l, while green tea contained approximately 2400 mg GAE/l (Rusak and others 2008). Extraction methods used are important to consider when comparing levels of components found in plant material. The phenolic content also did not change after autoclaving. This could be due to several reasons. Xu and others (2007) examined the phenolic content of citrus peel extract after heating (120 °C for 30, 60, 90 min) and the levels of free phenolic acids were found to increase whereas the ester, glycoside, and ester-bound forms were found to decrease. They also found that antioxidant activity increased after some heating, however, heating for too long (120 °C for 90 min) could destroy some phenolic compounds such as flavanone glycosides (Xu and others 2007). In our study, extracts were autoclaved at 121 °C for 30 min. Longer times may have resulted in destruction of phenolic compounds.

Higher concentrations of antimicrobials are typically required in food systems for protection than in less complex systems, such as microbiological media, to achieve the same levels of bacterial inactivation. Complex food systems contain components such as fats, proteins, and carbohydrates, which may interfere with antimicrobials. In the present study, the highest effective concentration of the lyophilized extract in a microbiological medium was used in milk. Extracts were less effective against *E. coli* O157:H7 1%, 2%, and whole milk compared to skim milk, while against *S. aureus*, in a lower concentration of extract, survived through 96 h in milk at all fat levels. This lack of activity may be attributed to the fat and protein components present in these media. In a previous study, the antimicrobial activity of essential oils from clove and cinnamon against *L. monocytogenes* was also shown to decrease when fat levels in milk were increased (Cava and others 2009). Similarly, lipids from corn oil were found to interfere with the antioxidant butylated hydroxyanisole, reducing the activity against bacteria such as *S. aureus* ATCC 12600 and *Pseudomonas fluoresens* ATCC 15456 (Rico-Munoz and Davidson 1983).

While pH 3.7 is a low pH in which bacterial pathogens often do not grow, in this study, growth of both *E. coli* O157:H7 and *S. aureus* was observed. This phenomenon could be explained by several factors. First, the components present in the milk, such as fat and proteins, may have provided a matrix to protect the bacteria. Further, milk is known to have buffering capacity. Heat processed milk (120 °C, 10 min) has been shown to have increased buffering capacity compared to milk that was not heat treated (Tamime 2009). Additionally, the pH may have recovered slightly after acid treatment or increased over time, allowing the bacteria to survive the stress and grow. Further, *E. coli* O157:H7 is more acid tolerant than other *E. coli* strains and in particular, strains ATCC 43889 and 43895 were found to be able to survive in apple cider for up to 21 days

(Miller and Kaspar 1994). These strains also survived in a pH 2 medium for 24 h with only a minimal drop in CFU/ml (Miller and Kaspar 1994). Uljas and Ingham (1998) found that when grown in TSB at pH 4.0, *E. coli* O157:H7 strain ATCC 43894 did not decrease at 4 or 21 °C. When grown in apple juice at pH 3.5, ATCC 43894 survived 7 days at 4 °C but did not survive at 21 °C. The strain used in our studies (ATCC 43894) was found to be more acid tolerant than ATCC 43889 (Uljas and Ingham 1998), and strain 'Cider' was isolated from an apple cider beverage, which can help to explain its acid tolerance. When grown in TSB with 0.6% yeast extract acidified with organic acids (acetic, citric, malic, lactic, or tartaric) to lower the pH, at 25 °C, *E. coli* O157:H7 increased by 2-4 log CFU/ml at pH ≥ 4 over a 56 day period (Connor and Kotrola 1995). Growth of *S. aureus* was also observed in the pH controls. It has been shown that *S. aureus* is acid tolerant to an extent and is able to grow in a wide pH range of 4.0-9.8 (Medvedova and Valik 2012). All of these factors combined may have contributed to the survival and growth of these pathogens in the pH 3.7 milk controls over 7 days.

Extracts of *H. sabdariffa* have the potential to be used as antimicrobials in a food beverage system. Because the calyces are extracted in water and *H. sabdariffa* is accepted as a natural flavoring substance by the U.S. Food and Drug Administration (21 CFR 172.510), its extracts might be good candidates to be applied to existing food and beverage systems as antimicrobials. In addition, activity has shown to be enhanced by autoclaving, enabling *Hibiscus* extracts to be added to a wide variety of foods prior to processing (i.e. pasteurization). Also, although autoclaving degraded anthocyanins, it did not adversely affect the phenolic content, which is important for antimicrobial activity. *Hibiscus* extracts have the potential to contribute additional health benefits due to the antioxidative properties and can be used as a natural alternative to

synthetic antimicrobials. Future work is needed to identify the active compounds. In addition, while milk was used as a model food in this study, it is not a practical beverage for *Hibiscus* extract treatment since the resulting milk will have a red color. More practical beverage applications for protection using *Hibiscus* extracts would be juice beverages. When *Hibiscus* extracts are added to, say, apple juice, red coloration could translate to a desirable aesthetic for consumers and could also be associated with enhanced safety against foodborne pathogens.

3.6 Acknowledgements

This research was supported by the Ivan Racheff Chair of Excellence endowment and the Tennessee Agricultural Experiment Station.

References

Burris KP, Davidson PM, Stewart Jr CN, Zivanovic S, Harte FM. 2012. Aqueous extracts of yerba mate (*Ilex paraguariensis*) as a natural antimicrobial against *Escherichia coli* O157:H7 in a microbiological medium and pH 6.0 apple juice. J Food Prot 75:753-7.

Cava R, Nowak E, Taboada A, Marin-Iniesta F. 2007. Antimicrobial activity of clove and cinnamon essential oils against *Listeria monocytogenes* in pasteurized milk. J Food Prot 70:2757-63.

Centers for Disease Control and Prevention. 2011. CDC estimates of foodborne illness in the United States. Available at: http://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html. Accessed 17 September 2013.

Chao C, Yin M. 2009. Antibacterial effects of roselle calyx extracts and protocatechuic acid in ground beef and apple juice. Foodborne Pathog Dis 6:201-6.

Christian KR, Jackson JC. 2009. Changes in total phenolic and monomeric anthocyanin composition and antioxidant activity of three varieties of sorrel (*Hibiscus sabdariffa*) during maturity. J Food Compos Anal 22:663-7.

Cisse M, Vaillant F, Acosta O, Dhuique-Mayer C, Dornier M. 2009. Thermal degradation kinetics of anthocyanins from blood orange, blackberry, and roselle using the arrhenius, eyring, and ball models. J Agric Food Chem 57:6285-91.

Connor DE, Kotrola JS. 1995. Growth and survival of *Escherichia coli* O157:H7 under acidic conditions. Appl Environ Microbiol 61:382-5.

Cowan MM. 1999. Plant products as antimicrobial agents. Clin Microbiol Rev 12:564-82.

Du CT, Francis FJ. 1973. Anthocyanins of roselle (*Hibiscus sabdariffa*, L.). J Food Sci 38:810-2.

Elsayed M, Elshafei A. 2011. Antimicrobial activity of roselle (*Hibiscus sabdariffa*) calyces and clove (*Syzygium aromaticum*) buds-water extracts using different extraction conditions. Adv Food Sci 33:141-5.

Giusti MM, Wrolstad RE. 2001. Characterization and measurement of anthocyanins by uv-visible spectroscopy, Unit F1.2.1-13. In: Wrolstad RE, editor. Current protocols in food analytical chemistry. New York: John Wiley & Sons.

Haslam E. 1998. Practical polyphenolics: from structure to molecular recognition and physiological action. Cambridge: Cambridge University Press. 438 p.

Jaroni D, Ravishankar S. 2012. Bactericidal effects of roselle (*Hibiscus sabdariffa*) against foodborne pathogens in vitro and on romaine lettuce and alfalfa sprouts. QAS 4:33-40.

Jay JM, Loessner MJ, Golden DA. 2005. Modern food microbiology. 7th ed. New York: Springer. 790 p.

Lee J, Durst RW, Wrolstad RE. 2005. Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: collaborative study. J AOAC Int 88:1269-78.

Liu KS, Tsao SM, Yin MC. 2005. In vitro antibacterial activity of roselle calyx and protocatechuic acid. Phytother Res 19:942-5.

Medvedova A, Valik L. 2012. *Staphylococcus aureus:* characterization and quantitative growth description in milk and artisanal raw milk cheese production. In: Amer Eissa A editor. Structure and function of food engineering, InTech, [serial online]. Available at: http://www.intechopen.com/books/structure-and-function-of-food- engineering. Accessed 9 July 2013. p. 71-102.

Miller LG, Kaspar CW. 1994. *Escherichia coli* O157:H7 acid tolerance and survival in apple cider. J Food Prot 57:460-4.

Montreau FR. 1972. On the analysis of total phenolic compounds in wines by the Folin–Ciocalteau method. Connaissance Vigne Vin 24:397-404.

Mounnissamy V, Kavimani S, Gunasegaran R. 2002. Antibacterial activity of gossypetin isolated from *Hibiscus sabdariffa.* Antiseptic 99:81-2.

Navarro García VM, Rojas G, Gerardo ZepedaL, Aviles M, Fuentes M, Herrera A, Jiménez E. 2006. Antifungal and antibacterial activity of four selected mexican medicinal plants. Pharm Biol 44:297-300.

Oboh G, Rocha JBT. 2008. Antioxidant and neuroprotective properties of sour tea (*Hibiscus sabdariffa*, calyx) and green tea (*Camellia sinensis*) on some pro-oxidant-induced lipid peroxidation in brain in vitro. Food Biophys 3:382-9.

Olaleye MT. 2007. Cytotoxicity and antibacterial activity of methanolic extract of *Hibiscus sabdariffa*. J Med Plants Res 1:9-13.

Ramirez-Rodrigues MM, Plaza ML, Azeredo A, Balaban MO, Marshall MR. 2011. Physicochemical and phytochemical properties of cold and hot water extraction from *Hibiscus sabdariffa*. J Food Sci 76:C428-35.

Ramírez-Rodrigues MM, Plaza ML, Azeredo A, Balaban MO, Marshall MR. 2012. Phytochemical, sensory attributes and aroma stability of dense phase carbon dioxide processed *Hibiscus sabdariffa* beverage during storage. Food Chem 134:1425-31.

Rico-Munoz E, Davidson PM. 1983. Effect of corn oil and casein on the antimicrobial activity of phenolic antioxidants. J Food Sci 48:1284-8.

Rusak G, Kones D, Likic S, Horzic D, Kovac M. 2008. Phenolic content and antioxidative capacity of green and white tea extracts depending on extraction conditions and the solvent used. Food Chem 110:852-8.

Scharff RL. 2012. Economic burden from health losses due to foodborne illness in the United States. J Food Prot 75:123-31.

Tamime A. 2009. Milk processing and quality management. West Sussex: Blackwell Publishing.

Tsai PJ, McIntosh J, Pearce P, Camden B, Jordan BR. 2002. Anthocyanin and antioxidant capacity in roselle (*Hibiscus sabdariffa* L.) extract. Food Res Int 35:351-6.

Uljas HE, Ingham SC. 1998. Survival of *Escherichia coli* O157:H7 in synthetic gastric fluid after cold and acid habituation in apple juice or trypticase soy broth acidified with hydrochloric acid or organic acids. J Food Prot 61:939-47.

Xu G, Ye X, Chen J, Liu D. 2007. Effect of heat treatment on the phenolic compounds and antioxidant capacity of citrus peel extract. J Agric Food Chem 55:330-5.

Appendix

Tables and figures

Table 3-1. Anthocyanin and phenolic content as well as polymerized color of commercially available *Hibiscus sabdariffa* calyx extracts

Hibiscus extract	Anthocyanin[a] concentration (mg/l) ± SE	Phenolic[b] concentration (mg/g) ± SE	Polymerized color (%)
Filtered (4 ºC)	14.27 ± 0.87	48.7 ± 2.9	52
Autoclaved (121 ºC, 30 min)	2.63 ± 0.04	53.8 ± 0.9	70

[a]Expressed as cyanidin-3-glucoside equivalents
[b]Expressed as gallic acid equivalents

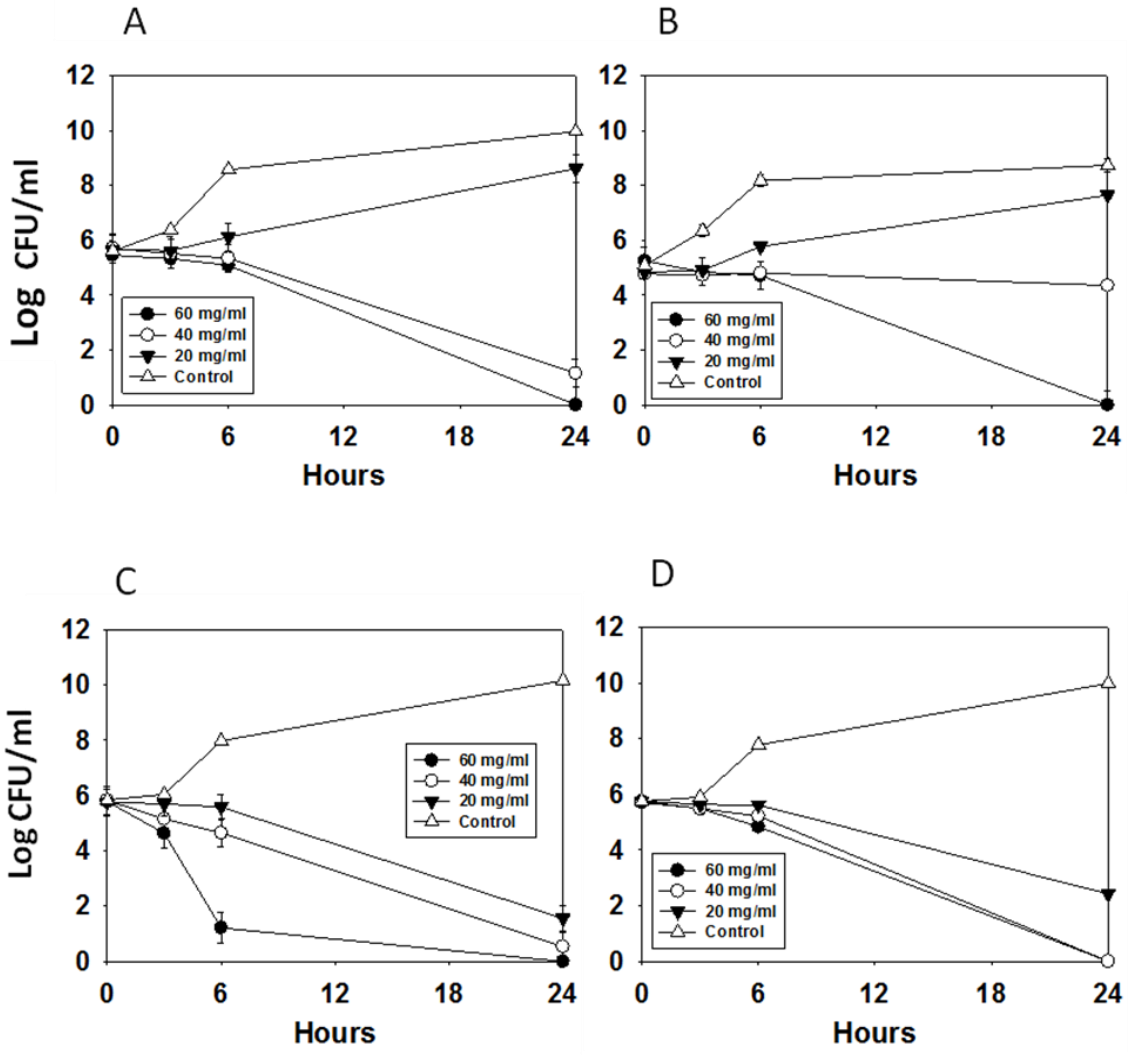

Figure 3-1. Antimicrobial activity of aqueous extracts filtered through a 0.22 µm membrane (A, B) and autoclaved extracts (C, D) from *Hibiscus sabdariffa* at concentrations of 0, 20, 40, and 60 mg/ml against *Escherichia coli* O157: H7 (A, C) strain 'Cider' and (B, D) strain ATCC 43894 after 0, 3, 6, and 24 h in tryptic soy broth at 37 °C. Error bars represent 95 % confidence intervals for the mean using least significance differences ($p < 0.05$).

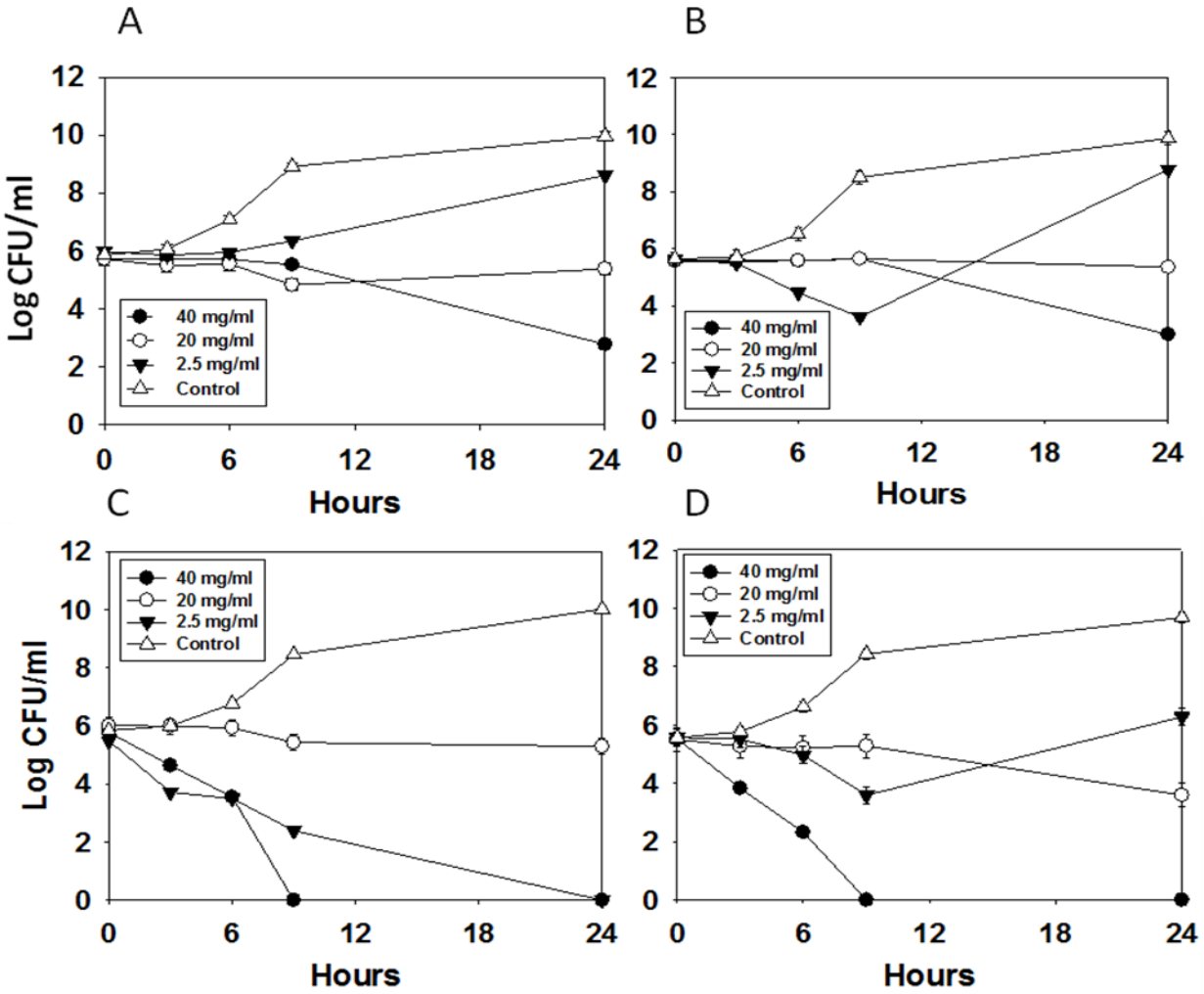

Figure 3-2. Antimicrobial activity of aqueous extracts filtered through a 0.22 µm membrane (A, B) and autoclaved extracts (C, D) from *Hibiscus sabdariffa* at concentrations of 0, 2.5, 20, 40 mg/ml against *Staphylococcus aureus* (A, C) strain SA 113 and (B, D) strain ATCC 27708 after 0, 3, 6, 9, and 24 h in tryptic soy broth at 37 °C. Error bars represent 95 % confidence intervals for the mean using least significance differences ($p < 0.05$).

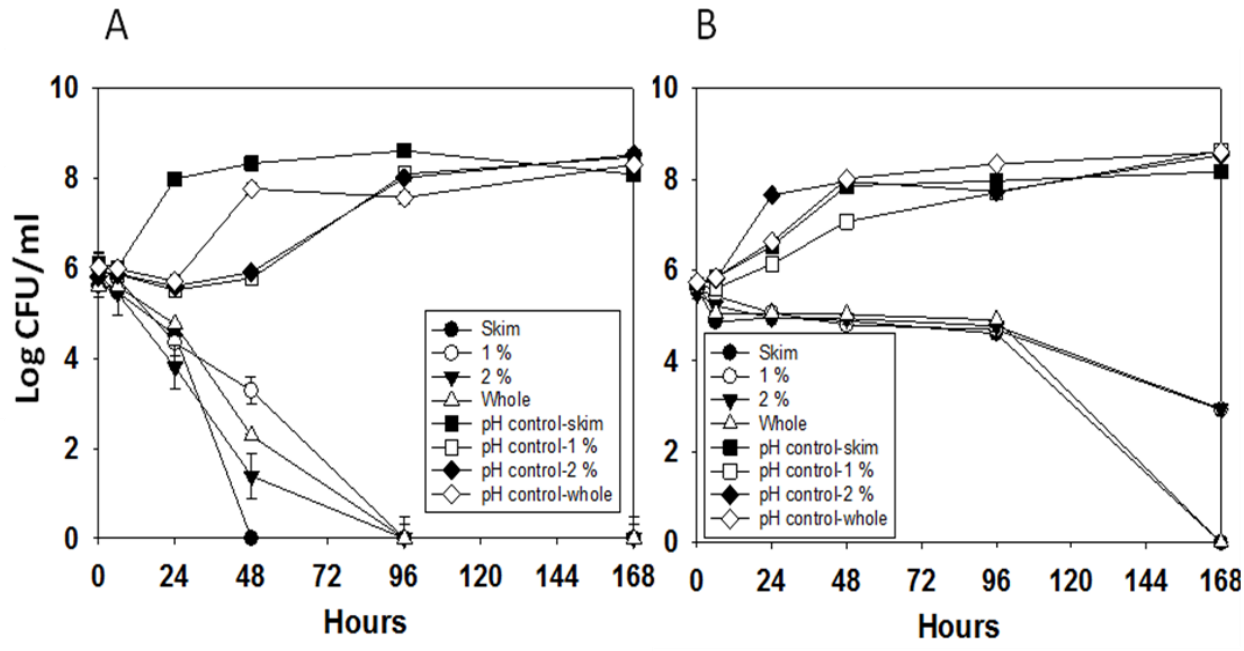

Figure 3-3. Antimicrobial activity of aqueous extracts from *Hibiscus sabdariffa* autoclaved at (A) 60 mg/ml (treatment) against a 1:1 mixture of *Escherichia coli* O157:H7 strains 'Cider' and ATCC 43894 and (B) 40 mg/ml (treatment) against a 1:1 mixture of *Staphylococcus aureus* 113 and ATCC 27708 in milk with various milkfat levels (skim, 1%, 2%, whole ultra high temperature (UHT)) after 0, 6, 24, 48, 96, and 168 h under room temperature storage conditions. The pH control used were water with no aqueous extracts (0 mg/ml) mixed with milk with the pH adjusted to ca. 3.7. Error bars represent 95 % confidence intervals for the mean using least significance differences ($p < 0.05$).

Chapter 4: Aqueous extracts of *Hibiscus sabdariffa* calyces as an antimicrobial rinse on hot dogs against *Listeria monocytogenes* and methicillin-resistant *Staphylococcus aureus*

Adapted from:

Kristen L. Higginbotham, Kellie P. Burris, Svetlana Zivanovic, P. Michael Davidson, C. Neal Stewart, Jr. Aqueous extracts of *Hibiscus sabdariffa* calyces as an antimicrobial rinse on hot dogs against *Listeria monocytogenes* and methicillin-resistant *Staphylococcus aureus*. Food Control. Submitted.

Kristen Higginbotham executed and analyzed the experiments, helped design the experiments and wrote the majority of the manuscript.

4.1 Abstract

Contamination by foodborne pathogens of ready-to-eat products is a major concern in the food industry. Novel methods to control foodborne pathogens are necessary due to continuing outbreaks as well as the development of antibiotic resistant pathogens. *Hibiscus sabdariffa* extracts could be a natural source for use as an antimicrobial rinse on ready-to-eat products to control pathogens. In this study, lyophilized *Hibiscus* flower extracts were examined for their antimicrobial activity as a rinse on all beef hot dogs against *Listeria monocytogenes* and methicillin-resistant *Staphylococcus aureus* (MRSA). Beef hot dogs were dip inoculated in overnight cultures of 1:1 mixtures of *L. monocytogenes* strains Scott A and 101 or MRSA strains ATCC 33591 and ATCC 33593 and were placed at 4°C overnight to allow for bacterial attachment. Hot dogs were rinsed with extracts (120, 240 mg/mL) or water (control) for 5, 15, 30, or 60 min and then plated immediately (0 h; no storage) or stored at 4°C overnight and plated at 24 h. Serial dilutions were plated in duplicate on both TSA and selection media, MOX (*Listeria*) or BP (MRSA), and the entire experiment was replicated 3 times. Higher extract concentrations, longer rinse times, and longer storage times were the most effective at inhibiting and/or killing *L. monocytogenes* and MRSA on hot dogs. *L. monocytogenes* was reduced to ca. 1.5 log CFU/g while MRSA was reduced to undetectable levels following rinsing of hot dogs with extracts at 240 mg/mL for 60 min and stored for 24 h. Both *L. monocytogenes* and MRSA were reduced ca. 2 log CFU/g following rinsing of hot dogs with extracts at 120 mg/mL for 60 min and stored for 24 h. This research demonstrates the effectiveness of *Hibiscus* extracts against *L. monocytogenes* and MRSA as an antimicrobial rinse on ready-to-eat meat products.

Key words: roselle, foodborne pathogens, frankfurters, bactericidal

4.2 Introduction

Listeria monocytogenes, a Gram-positive bacterium, is ubiquitous in nature and has a zero tolerance in foods in the United States according to the '*Listeria* rule' (9 CFR 430). *Listeria* can survive and grow at refrigeration temperatures and is difficult to eliminate once it is present in a processing facility. *L. monocytogenes* has been associated with foodborne illness outbreaks from a variety of foods, mostly of animal origin including dairy, meat products, and produce. In 1998, an outbreak of listeriosis occurred that was associated with hot dogs (Bil Mar Foods) which resulted in over 50 illnesses, 6 deaths, and 2 spontaneous abortions (CDC 1999). A study conducted by the CDC found that people 65 and older and pregnant women were about four and ten times more likely to be affected by *Listeria* respectively (CDC 2013). Therefore, it is critical that foods are as practically free of *Listeria* as possible to ensure the safety of consumers.

Staphylococcus aureus is a Gram-positive bacterium commonly found in the nose and on the skin of animals and people. Antibiotics are widely used in food animal production for disease prevention. This use has contributed to an increase globally in multi-drug resistant bacteria. While methicillin-resistant *Staphylococcus aureus* (MRSA) has been primarily considered a healthcare associated pathogen, it has been associated with and detected in food products, especially meats. In a recent study in the United States, Waters and others (2011) examined the contamination of meats and poultry by *S. aureus* and found 37% contamination (14/38) in beef products. For all of the meat samples tested, 52% of the *S. aureus* isolates detected were multi-drug resistant (Waters and others 2011). Further, the rates of animal to human transmission of MRSA are increasing. In Denmark, people who lived or worked on hog farms were more likely to be colonized or infected with MRSA CC398 and the hogs were identified as the source of infection (Lewis and others 2008). MRSA, just as methicillin-susceptible *S. aureus*, is capable of

producing enterotoxins, which could cause food intoxication if the toxins were consumed. A foodborne illness outbreak occurred in the United States in which the cause was staphylococcal foodborne illness, specifically MRSA, from consuming barbeque pork and cole slaw purchased from a delicatessen (Jones and others 2002).

As the trend for 'natural products' consumption grows, alternatives to chemical antimicrobials and sanitizers added to ready-to-eat products need to be discovered in efforts to reduce and eliminate pathogens from contaminating the food supply. A variety of plant extracts have been shown to provide antimicrobial activities (Cowan 1999). *Hibiscus sabdariffa* is a subtropical plant species grown in countries such as India, Mexico, and Thailand. Calyces from this plant are often used to prepare a tart beverage (Hibiscus tea) that is deeply red in color, often consumed for its medicinal benefits. The calyces of *Hibiscus* have known antimicrobial activity against *S. aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Bacillus cereus*, *Propionibacterium acnes*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Salmonella*, and *L. monocytogenes* (Chao and Yin 2009; Chomnawang and others 2009; Liu and others 2005; Olaleye 2007). The minimum inhibitory concentration (MIC) for an aqueous *H. sabdariffa* extract against *L. monocytogenes* was found to be 136 ± 24 µg/mL and 84 ± 8 µg/mL for an ethanol extract (Chao and Yin 2009). Against MRSA, an ethanol extraction of *H. sabdariffa* was found to have an MIC of 5000 µg/mL (Chomnawang and others 2009). The chemical composition of the bioactive calyces have been partially identified and have been shown to contain numerous compounds that may be contributing to the observed antimicrobial activity, such as organic acids, phenolic acids, alkaloids, and anthocyanins (Christian and Jackson 2009; Olaleye 2007; Tsai and others 2002). Gossypetin and protocatechuic acid are two compounds in particular that have been shown to have antimicrobial activity (Mounnissamy and others 2002;

Chao and Yin 2009). Protocatechuic acid content in an aqueous *Hibiscus* extract was determined to be 2.8 ± 0.7 mg/g (Chao and Yin 2009).

Often, ready-to-eat products are formulated with antimicrobial agents to inhibit the growth of pathogens. Lactates and diacetates are frequently added to the product formulation (USDA 2012); however, their use alone has been shown to be ineffective over time to inhibit the growth of *Listeria* (Perumalla and others 2013). Therefore, combination treatments such as lowering the water activity of the product or changing the pH and including additional post-processing treatments such as steam pasteurization can be used to assist in inhibiting pathogens such as *Listeria* (USDA 2012). Despite all of these methods used, outbreaks continue to occur. Hot dogs and other ready-to-eat products can become contaminated after the heating process but before they are packaged. In order to prevent outbreaks and/or intoxications from pathogens such as *Listeria* and MRSA, an antimicrobial rinse can be applied before or after heating. *Hibiscus sabdariffa* calyx extracts would be a natural option for food manufacturers and could be used in place of or in addition to processes and antimicrobial agents such as the diacetates and lactates that are currently used. The purpose of the present study was to demonstrate the effectiveness of *Hibiscus* calyx extracts as an antimicrobial rinse on hot dogs against *L. monocytogenes* and MRSA, two pathogens of concern in ready-to-eat meats.

4.3 Materials and Methods

4.3.1 Preparation of extract

USDA organic *H. sabdariffa* flowers (calyces) were purchased from Starwest Botanicals (SKU: 209355-31, Sacramento, CA). Extracts were prepared according to published methods (Burris

and others 2012) with modifications. Calyces were ground using a blender (Oster, Boca Raton, FL) to less than 1 mm and extracted using water at a ratio of 1 g of tissue to 3.6 mL water for 2 h at 4°C to limit microbial growth in the dark with periodic shaking. Extracts were then centrifuged at 5000 x g for 30 min and filtered through miracloth (EMD Millipore, Billerica, MA) to remove large insoluble particles. Extracts were placed at -80°C and freeze dried using LabConco FreeZone 12 Liter Freeze Dry System (Kansas City, MO) to concentrate and stored in sealed containers until testing.

4.3.2 Phenolic content

Phenolic content of the extract was determined using Folin-Ciocalteu's phenol reagent (Montreau 1972). Extracts were rehydrated in water (1 mg/mL) and autoclaved at 121°C for 30 min. Extracts were then filtered through Whatman No. 4 after cooling. The phenolic content was quantified using gallic acid as the standard and expressed in mg gallic acid equivalents (GAE)/g dry calyx extract after the absorbance was read at 765 nm.

4.3.3 Culture preparation

Stock cultures of *L. monocytogenes* Scott A and 101 were obtained from the Department of Food Science and Technology and MRSA strains ATCC 33591 and ATCC 33593 were purchased from the American Type Culture Collection (Manassas, VA). Cultures were grown in tryptic soy broth (TSB; Becton, Dickinson and Co., Sparks, MD) and stored in glycerol at -20°C. Working cultures were obtained by inoculating 50 mL TSB with 200 µL stock cultures and incubating for 24 h at 32°C for *L. monocytogenes* and at 35-37°C for MRSA and each strain was subcultured once.

4.3.4 Hot dog rinse

Beef hot dogs (Kroger Value brand; The Kroger Co., Cincinnati, OH) were inoculated in overnight cultures of 1:1 mixtures of *L. monocytogenes* strains Scott A and 101 or MRSA strains ATCC 33591 and ATCC 33593 (25 mL of each strain for 2 hot dogs) in sterile Whirl-Pak stomacher bags (Nasco, Fort Atkinson, WI). Stomacher bags containing hot dogs were placed at 4°C overnight to allow for bacterial attachment and were then cut using a sterile blade into 4 equal pieces. Eight pieces per stomacher bag were used and 60 mL of treatments were added (control: water, treatment: *Hibiscus* extract autoclaved for 30 min at 121°C at the concentrations of 120 and 240 mg/mL). The following rinse times were used: 5, 15, 30, and 60 min, at which time, 2 pieces of hot dog were removed and placed separately into sterile stomacher bags. One piece was used to plate 0 h (no storage) while the other hot dog piece was put at 4°C overnight, held for 24 h, and plated (24 h storage). For plating, 90 mL of 0.1 % peptone was added to the bag and stomached for 30 s at 230 rpm. Serial dilutions were plated in duplicate on TSA and MOX (*Listeria*) or BP (MRSA) and incubated for 48 h at 32°C for *Listeria* and 24 h at 37°C for MRSA. Each experiment was replicated 3 times.

4.3.5 Statistical analysis

A randomized complete block design was used, blocking on replicate. Split-plot was the treatment design, with extract applied to the whole bag of hot dog pieces and then time and storage treatment factors applied to the individual pieces. Results were analyzed using ANOVA, using a generalized linear model (SAS 9.3, Cary, NC). Least significant differences were used to determine significant differences among treatment means ($p<0.05$).

4.4 Results

For every 100 g of ground calyces used, approximately 21 g of freeze dried extract was obtained. The lyophilized extract was also easily re-hydrated in water. The extract had a pH of approximately 2.5, and the phenolic content was determined to be ca. 16 mg GAE/g freeze-dried extract.

Extracts were more effective at reducing bacterial growth than water for use as a rinse on hot dogs, and extracts at 240 mg/mL were more effective than 120 mg/mL against both *L. monocytogenes* and MRSA (Tables 1 & 2). Also, for both extract concentrations, increasing rinse times and storage times resulted in significant reductions for both *L. monocytogenes* and MRSA. Against *L. monocytogenes*, on TSA, all interactions were significant ($p<0.05$) with the exception of the storage and rinse time interaction ($p=0.34$) and the extract, storage, and rinse time interaction ($p=0.06$). On MOX, all interactions were significant except for the storage and rinse time interaction ($p=0.45$). Against MRSA, for both types of media, all interactions were significant ($p<0.05$). Overall, higher extract concentrations, longer rinse times, and longer storage times were the most effective at inhibiting and/or killing both *L. monocytogenes* and MRSA on hot dogs.

4.5 Discussion

In the present study, *Hibiscus* extracts heated in steam at 121°C for 30 min were examined for their antimicrobial activity as a rinse on hot dogs to inhibit and/or inactivate the foodborne pathogens, *L. monocytogenes* and MRSA. In a previous study we performed, autoclaved extracts

exhibited enhanced antimicrobial activity compared to filtered extracts against *S. aureus* and *Escherichia coli* O157:H7 (Higginbotham and others 2013). The population of *L. monocytogenes* was reduced when rinsed in the extract but was not eliminated completely. A longer rinse time, a higher concentration of extract, or a longer storage time may have reduced the bacterial population further. Chao and Yin (2009) observed that higher concentrations of *Hibiscus* (10 mg compared to 5 mg) added to ground beef (100 g) had greater antimicrobial activity against foodborne pathogens, such as *L. monocytogenes* and *S. aureus*. Extracts were also more effective against MRSA than *Listeria*. These results could be explained due to storage of the hot dogs at 4°C following rinse treatments. *Listeria* is able to grow at refrigeration temperatures, with a minimum growth temperature of 1.7 ± 0.5°C (Junttila and others 1988). Cole and others (1990) examined *L. monocytogenes* strain ATCC 19115 and found that at lower temperatures of 5 and 10°C, the pathogen had a greater ability to survive higher salt and lower pH conditions than when kept at 30°C. Borezee and others (2000) determined that the *oppA* gene contributed in the transportation of oligopeptides necessary for growth when *L. monocytogenes* was grown at lower temperatures. Additionally, Annous and others (1997) observed that *L. monocytogenes* will change its fatty acid composition, either by shortening its fatty acids or changing the branching from iso to anteiso at lower temperatures to maintain fluidity in the membrane. In contrast, *S. aureus* can survive at lower temperatures, but typically does not grow. Notermans and Heuvelman (1983) reported that *S. aureus* was able to survive but not grow at 8°C and enterotoxins were not produced. Similarly, Schmitt and others (1990) looked at *S. aureus* strains isolated from foods and no growth was observed at 9.5°C and its minimum growth temperature was determined to be 11°C.

In the present study, compared to the control, *L. monocytogenes* was reduced an additional ca. 3.7 log CFU/g, when rinsed with *H. sabdariffa* extracts (240 mg/mL) for 60 min. Numerous other methods to inactivate pathogens on ready-to-eat products have been examined. Cetylpyridinium chloride (CPC) at 1% reduced *L. monocytogenes* on frankfurters ca. 1.4 to 1.7 log CFU/g after spraying (Singh and others 2005). However, CPC is a quaternary ammonium compound and thus an *Hibiscus* rinse could be used in products claiming to be all natural. A similar study found that a spray consisting of a combination of 5% lactic acid and 0.5% sodium lauryl sulfate applied to frankfurters after being inoculated with *L. monocytogenes* resulted in a reduction of 2.8 ± 0.2 log CFU/cm^2 after being stored for 90 days (Byelashov and others 2008). Compared to the present study, after only 24 h, an ca. 3.7 log CFU/g and greater than 5 log CFU/g reduction was observed for *L. monocytogenes* and MRSA, respectively, however storage times extending beyond 24 h were not examined. Essential oils of thyme and clove at 5 mL/L resulted in reductions of 0.67-1.05 and 1.15-1.71 log CFU/g respectively of *L. monocytogenes* on hot dogs where storage time was not a factor (Singh and others 2003). However, essential oils are known for their pungent odor and taste and may result in undesirable sensory effects, especially at levels necessary to see comparable reductions to our *Hibiscus* rinse. Singh and others (2003) also found that increasing the fat content of the hot dog resulted in decreased effectiveness of the essential oils. In the present study, the fat content of the hot dogs was 28.5%, which would be considered a full-fat hot dog. Another option to control pathogens in hot dogs, which was recently studied, demonstrated the effects of adding natural extracts of green tea (0.35%) and grape seed (0.22%) along with two antimicrobial preservatives, potassium lactate (1.5%) and sodium diacetate (0.15%) and found that growth of *L. monocytogenes* was inhibited over a 28 d period, but the use of the antimicrobial preservatives alone were not as effective

(Perumalla *et al*., 2013). The hot dogs used in the present study contained potassium lactate, sodium lactate, sodium diacetate, and sodium nitrite, which may have acted additively or synergistically with our *Hibiscus* extracts to further inhibit the growth of *L. monocytogenes* and MRSA.

Here, we examined calyx extracts as a rinse on hot dogs, but *Hibiscus* extracts also have the potential to be used as rinses on other types of foods such as fresh produce. Jaroni and Ravishankar (2012) showed that growth of *Escherichia coli* O157:H7 on romaine lettuce and *Salmonella* on alfalfa sprouts were both reduced ca. 4 log CFU/g when rinsed with aqueous *H. sabdariffa* calyx extracts (100% v/v). An *in vitro* study was conducted where it was observed *L. monocytogenes* required longer incubation times of between 48 and 72 h to see inactivation compared to 24 h for *E. coli* and *Salmonella* and the extracts were also more effective at 25°C compared to 4 and 8°C for *L. monocytogenes* (Jaroni and Ravishankar 2012). Similarly, in this study, *L. monocytogenes* was not inactivated completely and temperature could be contributing to survival.

The concern for ready-to-eat products often comes from post-process contamination, where pathogens are passed to the food product through cross contamination from processing equipment. Industry uses standard guidelines to prevent the growth of pathogens such as *Listeria* in foods. There are three alternatives in place that can be used to control *Listeria* (USDA 2012). Alternative 1 includes using both an antimicrobial agent and a post-lethality treatment while Alternative 2 uses one or the other and Alternative 3 uses sanitation to control *Listeria* (USDA 2012). In this study, the antimicrobial extract rinse could be included in either Alternative 1 or 2

(USDA 2012). Even though *Listeria* was not eliminated completely, these *Hibiscus* extracts could be used along with other strategies to control this pathogen in ready-to-eat meats. Extracts may also be more effective after longer storage times. Also, since the color of the hot dogs rinsed in extracts was observed to be redder in color, sensory analysis would need to be performed to ensure consumer acceptance.

4.6 Conclusions

With increasing variants of multi-drug resistant bacteria and an increasing aging population more susceptible to the consequences of foodborne pathogens, the need for alternative compounds to control these bacteria in foods is required. Aqueous extracts of *H. sabdariffa* calyces were effective against both *L. monocytogenes* and MRSA as a rinse on hot dogs. *H. sabdariffa* extracts could be used as a natural alternative to traditional antimicrobial preservatives to prevent growth and/or eliminate pathogens in food products, especially since the calyces are extracted in water, and *Hibiscus* is considered generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (21 CFR 172.510). Extracts were also effective after heating in an autoclave, demonstrating their stability and potential use before or after heat processing of foods.

4.7 Acknowledgements

This research was supported by the Ivan Racheff Chair of Excellence endowment and the University of Tennessee Agricultural Experiment Station.

References

Annous BA, Becker LA, Bayles DO, Labeda DP, Wilkinson BJ. 1997. Critical role of anteiso-$C_{15:0}$ fatty acid in the growth of *Listeria monocytogenes* at low temperatures. Appl Environ Microbiol 63:3887-94.

Borezee E, Pellegrini E, Berche P. 2000. OppA of *Listeria monocytogenes*, an oligopeptide-binding protein required for bacterial growth at low temperature and involved in intracellular survival. Infect Immun 68:7069-77.

Burris KP, Davidson PM, Stewart Jr CN, Zivanovic S, Harte FM. 2012. Aqueous extracts of yerba mate (*Ilex paraguariensis*) as a natural antimicrobial against *Escherichia coli* O157:H7 in a microbiological medium and pH 6.0 apple juice. J Food Prot 75:753-7.

Byelashov OA, Kendall PA, Belk KE, Scanga JA, Sofos JN. 2008. Control of *Listeria monocytogenes* on vacuum-packaged frankfurters sprayed with lactic acid alone or in combination with sodium lauryl sufate. J Food Prot 71:728-34.

Centers for Disease Control and Prevention. 1999. Update: multistate outbreak of listeriosis—United States, 1998-1999. MMWR Weekly 47:1117-8.

Centers for Disease Control and Prevention. 2013. *Listeria* (listeriosis). Available at: http://www.cdc.gov/listeria/risk.html. Accessed: September 20, 2013.

Chao CY, Yin MC. 2009. Antibacterial effects of roselle calyx extracts and protocatechuic acid in ground beef and apple juice. Foodborne Path Dis 6:201-6.

Chomnawang MT, Surassmo S, Wongsariya K, Bunyapraphatsara N. 2009. Antibacterial activity of Thai medicinal plants against methicillin-resistant *Staphylococcus aureus*. Fitoterapia 80:102-4.

Christian KR, Jackson JC. 2009. Changes in total phenolic and monomeric anthocyanin composition and antioxidant activity of three varieties of sorrel (*Hibiscus sabdariffa*) during maturity. J Food Compos Anal 22: 663-7.

Cole MB, Jones MV, Holyoak C. 1990. The effect of pH, salt concentration and temperature on the survival and growth of *Listeria monocytogenes*. J Appl Microbiol 69:63-72.

Cowan MM. Plant products as antimicrobial agents.1999. Clin Microbiol Rev 12:564-82.

Higginbotham KL, Burris KP, Zivanovic S, Davidson PM, Stewart Jr. CN. 2013. Antimicrobial activity of *Hibiscus sabdariffa* aqueous extracts against *Escherichia coli* O157:H7 and *Staphylococcus aureus* in a microbiological medium and milk of various fat concentration. J Food Prot (*in press*).

Jaroni D, Ravishankar S. 2012. Bactericidal effects of roselle (*Hibiscus sabdariffa*) against foodborne pathogens *in vitro* and on romaine lettuce and alfalfa sprouts. QAS 4:33-40.

Jones TF, Kellum ME, Porter SS, Bell M, Schaffner W. 2002. An outbreak of community-acquired foodborne illness caused by methicillin-resistant *Staphylococcus aureus*. Emerg Infect Dis 8:82-4.

Junttila JR, Niemela SI, Hirn J. 1988. Minimum growth temperatures of *Listeria monocytogenes* and non-haemolytic *Listeria*. J Appl Bacteriol 65:321-7.

Kramer A, Schwebke I, Kampf G. 2006. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. BMC Infect Dis 6:130.

Kluytmans JAJW. 2010. Methicillin-resistant *Staphylococcus aureus* in food products: cause for concern or case for complacency? Clinical Microbiol Infect 16:11-5.

Lewis HC, Molbak K, Reese C, Aarestrup FM, Selchau M, Sorum M, Skov RL. 2008. Pigs as source of methicillin-resistant *Staphylococcus aureus* CC398 infections in humans, Denmark. Emerg Infect Dis 14:1383-9.

Liu KS, Tsao SM, Yin MC. 2005. *In vitro* antibacterial activity of roselle calyx and protocatechuic acid. Phytother Res 19:942-5.

Montreau FR. 1972. On the analysis of total phenolic compounds in wines by the Folin–Ciocalteau method. Connaissance Vigne Vin 24:397-404.

Mounnissamy V, Kavimani S, Gunasegaran R. 2002. Antibacterial activity of gossypetin isolated from *Hibiscus sabdariffa.* Antiseptic 99:81-2.

Olaleye MT. 2007. Cytotoxicity and antibacterial activity of methanolic extract of *Hibiscus sabdariffa*. J Med Plants Res 1: 9-13.

Perumalla AVS, Hettiarachchy NS, Over K, Ricke SC, Slavik MF, Gbur E, Davis B, Acosta S. 2013. Effect of partial replacement of potassium lactate and sodium diacetate by natural green tea and grape seed extracts and postpackaging thermal treatment on the growth of *Listeria monocytogenes* in hotdog model system. Int J Food Sci Tech 48:918-26.

Schmitt M, Schuler-Schmid U, Schmidt-Lorenz W. 1990. Temperature limits of growth, TNase and enterotoxin production of *Staphylococcus aureus* strains isolated from foods. Int J Food Microbiol 11:1-20.

Singh A, Singh RK, Bhunia AK, Singh N. 2003. Efficacy of plant essential oils as antimicrobial agents against *Listeria monocytogenes* in hotdogs. LWT 36:787-94.

Singh M, Gill VS, Thippareddi H, Phebus RK, Marsden JL, Herald TJ, Nutsch AL. 2005. Antimicrobial activity of cetylpyridinium chloride against *Listeria monocytogenes* on frankfurters and subsequent effect on quality attributes. J Food Prot 68:1823-30.

Tsai PJ, McIntosh J, Pearce P, Camden B, Jordan BR. 2002. Anthocyanin and antioxidant capacity in roselle (*Hibiscus sabdariffa* L.) extract. Food Res Int 35:351-6.

United States Department of Agriculture. 2012. FSIS compliance guideline: controlling *Listeria monocytogenes* in post-lethality exposed ready-to-eat meat and poultry products. Available at: http://www.fsis.usda.gov/shared/PDF/Controlling_LM_RTE_guideline_0912.pdf. Accessed 25 August 2013.

Waters AE, Contente-Cuomo T, Buchhagen J, Liu CM, Watson L, Pearce K, Foster JT, Bowers J, Driebe EM, Englethaler DM, Keim PS, Price LB. 2011. Multidrug-resistant *Staphylococcus aureus* in US meat and poultry. CID 52:1227-30.

Appendix

Table 4- 1. Log CFU/g[a], plated on non-selective tryptic soy agar (TSA) and selective Modified Oxford (MOX) media, of *Listeria monocytogenes* (mixture of 1:1 of strains 101 and Scott A) following a rinse (5, 15, 30, 60 min) with either water (0 mg/mL) or *Hibiscus* extract (120 or 240 mg/mL) to inoculated hot dogs and stored at 4°C for 0 or 24 h

		Control		*Hibiscus* extract (120 mg/mL)		*Hibiscus* extract (240 mg/mL)	
		Storage Time (h)					
	Rinse Time (min)	0	24	0	24	0	24
TSA	5	5.67A	5.44AB	5.61A	5.35AB	5.74A	4.81CD
	15	5.47AB	5.64A	5.66A	5.04BC	5.62A	4.10FG
	30	5.53A	5.47AB	4.75CDE	4.55DEF	4.39EF	3.17I
	60	5.53A	5.29AB	3.84GH	3.68GH	3.41HI	1.62J
MOX	5	5.59A	5.32ABCD	5.52ABC	5.34ABCD	5.61AB	4.58FG
	15	5.27ABCD	5.34ABCD	5.33ABCD	5.06DF	5.33ABCD	3.77HI
	30	4.99DEF	5.12CDE	4.68EG	4.05H	4.15H	2.96J
	60	5.19BCD	5.23ABCD	3.82H	3.40IJ	3.23J	1.41K

[a]Log CFU/g values in rows and columns, separated by media type, not followed by a like letter are significantly different ($p<0.05$).

Table 4- 2. Log CFU/g[a], plated on non-selective tryptic soy agar (TSA) and selective Baird-Parker (BP) media, of methicillin-resistant *Staphylococcus aureus* (mixture of 1:1 of strains ATCC 33591 and ATCC 33593) following a rinse (5, 15, 30, 60 min) with either water (0 mg/mL) or *Hibiscus* extract (120 or 240 mg/mL) to inoculated hot dogs and stored at 4°C for 0 or 24 h

		Control		*Hibiscus* extract (120 mg/mL)		*Hibiscus* extract (240 mg/mL)	
		Storage Time (h)					
	Rinse Time (min)	0	24	0	24	0	24
TSA	5	5.64^{AB}	5.38^{AB}	5.71^{AB}	5.67^{AB}	5.61^{AB}	5.27^{AB}
	15	5.48^{AB}	5.61^{AB}	5.79^{A}	5.21^{B}	5.38^{AB}	4.21^{C}
	30	5.58^{AB}	5.35^{AB}	4.56^{C}	4.66^{C}	4.61^{C}	2.49^{E}
	60	5.52^{AB}	5.59^{AB}	4.57^{C}	3.50^{D}	4.63^{C}	ND^{F}
BP	5	5.71^{A}	5.43^{ABCD}	5.81^{A}	5.55^{ABC}	5.68^{AB}	5.28^{ABCDE}
	15	5.33^{ABCD}	5.38^{ABCD}	5.79^{A}	5.44^{ABCD}	5.60^{ABC}	4.31^{G}
	30	5.63^{ABC}	5.36^{ABCD}	5.05^{BCDEF}	4.66^{EFG}	4.89^{DEF}	2.37^{I}
	60	5.56^{ABC}	5.67^{AB}	5.02^{CDEF}	3.42^{H}	4.52^{FG}	ND^{J}

[a]Log CFU/g values in rows and columns, separated by media type, not followed by a like letter are significantly different ($p<0.05$)
ND = Below detection level of 10 CFU/g

Chapter 5: Chemical composition and antimicrobial activity of greenhouse grown and commercially acquired *Hibiscus sabdariffa* calyces

5.1 Abstract

Hibiscus sabdariffa is a tropical shrub species commonly cultivated for its edible calyces. Varieties of *Hibiscus* vary in color, size, acidity, and compounds. In this study chemical composition and antimicrobial activity of calyces grown in the greenhouse from seed (Roselle, Thai Red, Jamaican Cocktail Red) were compared to calyces acquired commercially (brands 'Starwest Botanicals', 'El Girasol', and 'Saafara'). Lyophilized extracts of the calyces were used to determine the anthocyanin and phenolic content. Antimicrobial activity was evaluated against *Staphylococcus aureus* strain ATCC 27708 and the effect of native pH (2.5) compared to extract adjusted to pH 7 was analyzed. Phenolic content as determined by Folin-Ciocalteu remained similar after autoclaving (121°C, 15 min) while monomeric anthocyanin content decreased in all extracts. The pH was found to influence antimicrobial activity. *S. aureus* was inactivated after 3 h when exposed to extracts (60 mg/ml) at native pH 2.5 while there was generally only a bacteriostatic effect on growth over 48 h at pH 7. Antimicrobial activity was different for greenhouse grown calyces compared to those acquired commercially. Thai Red and Jamaican Cocktail Red extracts at pH 7 were inhibitory through 9 h against *S. aureus* but then recovered by 24 h. Roselle extracts were most similar in antimicrobial activity to the commercial varieties. Overall, differences in antimicrobial activity and phenolic content were observed between greenhouse grown and commercial calyces. Commercial calyces were more effective against *S. aureus* and had higher levels of phenolic compounds.

5.2 Introduction

Plant products are good candidates for their antimicrobial and medicinal properties because of their high levels of bioactive compounds. *Hibiscus sabdariffa* (family Malvaceae) is a shrub that grows best in subtropical and tropical conditions (Morton 1987). Calyces from this plant are harvested approximately 2-3 weeks after flowering, afterwhich the fruit inside is removed, and calyces are subsequently dried and packaged (Cisse and others 2009). The calyces are typically used to make a tart beverage that is served either hot or cold and often consumed for its medicinal benefits. However, the calyces have also been used to prepare jams and jellies, added as a topping to salads, or as a coloring additive (Morton 1987). Many varieties of this shrub exist and calyces often vary in color, size, and chemical content. Calyces can vary in degree of red color or can be completely free of anthocyanins and exhibit a green or white color.

Hibiscus calyces have previously been examined for chemical composition and numerous compounds such as organic acids, anthocyanins, phenolic acids, quercetin, gossypetin, alkaloids, and saponins have been identified (Ramirez-Rodrigues and others 2011, Hirunpanich and others 2005, Olaleye 2007). The flavonoid gossypetin as well as the phenolic acid, protocatechuic acid have been shown to exhibit antimicrobial activity (Mounnissamy and others 2002, Liu and others 2005, Chao and Yin, 2009). The protocatechuic acid content has been examined in dried aqueous extracts of calyces and was determined to be 2.8 ± 0.7 mg/g (Chao and Yin 2009).

The objective of this study was to examine and compare the chemical composition and antimicrobial activity of calyces grown in the greenhouse from seed (Roselle, Thai Red,

Jamaican Cocktail Red) to calyces commercially acquired (brands 'Starwest Botanicals', 'El Girasol', and 'Saafara').

5.3 Materials and methods

5.3.1 Culture preparation

Staphylococcus aureus strain ATCC 27708 was stock culture obtained from the Center for Environmental Biotechnology, University of Tennessee, Knoxville. All cultures were grown in tryptic soy broth (TSB; Difco, Sparks, MD) and stored in glycerol at -20°C. Working cultures were obtained by inoculating 200 µl stock cultures in 50 ml TSB and incubating for 24 hr at 35-37°C and subcultured once. After incubation, cultures were diluted to ca. 5.0-6.0 log CFU (colony forming units)/ml and tested for antimicrobial activity.

5.3.2 Growth of *Hibiscus* calyces

Hibiscus sabdariffa varieties ('Roselle', 'Jamaican Cocktail Red', and 'Thai Red') were grown from seed in the greenhouse. Approximately 30 plants of each variety were planted. Plants were maintained at ca. 30 °C and day length was 16 h. Flowering occurred approximately 3-5 months after germination. Calyces were collected about 3 wk after flowering, fruits were removed, and calyces were dried artificially at 50-60 °C for 24 h and stored at room temperature until extraction.

5.3.3 Aqueous extraction

Both commercially acquired calyces and greenhouse grown varieties were used for extraction. The commercial brands used were: 'Starwest Botanicals' (Starwest Botanicals, SKU: 209355-31, Sacramento, CA), 'El Girasol' and 'Mi Costenita' purchased from a local grocery store (Knoxville, TN). Dried calyces of commercial and greenhouse grown *Hibiscus* were ground using a blender (Oster, Boca Raton, FL) to a particle size < 1 mm and then extracted with water at a ratio of 1 g tissue for every 5 ml water for the greenhouse grown varieties and 1 g tissue for every 3.6 ml water for the commercial varieties. Extracts were allowed to stand for 2 h at room temperature with periodic shaking every 15-30 min. Supernatant was poured through miracloth (EMD Millipore, Billerica, MA) to remove large particles. Extracts were then frozen at -80 °C and then freeze dried using LabConco FreeZone 12 Liter Freeze Dry System (Kansas City, MO) to concentrate. Extracts were then stored in a sealed container at room temperature until ready to test.

5.3.4 Determination of Color

Color was determined using the Hunter Lab colorimeter Miniscan XE Plus (Hunter Associates Laboratory, Reston, VA). L, a, and b values were determined for all *Hibiscus* extracts at the concentration of 5 mg/ml. Samples were placed in water activity cups and measured. The L value is from 0 to 100 with 0 being black and 100 being white. A positive a-value is red whereas a negative a-value is green. A positive b-value is yellow and a negative b-value is blue.

5.3.5 Anthocyanin and phenolic content

The pH differential method (Lee and others 2005) was used to determine the monomeric anthocyanin content present in the extracts (sterilized by membrane filtration (0.22 µm) or heating by autoclave at 121°C for 30 min) at the concentration of 1 mg/ml. Absorbance was read at 520 nm and 700 nm at pH 1.0 and 4.5 with a dilution factor of 3 expressed as mg cyanidin-3-glucoside equivalents/l (MW = 449.2, ε = 26,900). Folin-Ciocalteu's phenol reagent was used to determine phenolic content (Montreau 1973). Rehydrated extracts (1 mg/ml) were either filtered through 0.22 µm or autoclaved. Extracts were then filtered through Whatman No. 4 after cooling to remove any particulates. The phenolic content was quantified using gallic acid as the standard, and expressed in mg gallic acid equivalents (GAE)/g dry calyx extract after the absorbance was read at 725 nm.

5.3.6 Time-kill assays

Lyophilized extracts were resuspended in 0.1 M phosphate buffer pH 7.2 and pH was subsequently adjusted to approximately pH 7 using sodium hydroxide pellets (Fisher Scientific, Waltham, MA). Extracts were also resuspended in MilliQ water (10 ml) and tested at their native pH of 2.5. All extracts were autoclaved at 121 °C for 30 min to sterilize. Extracts at a final concentration of 60 mg/ml were added to 12.5 ml TSB and 2.5 ml diluted *S. aureus* ATCC 27708 and incubated at 35-37 °C over 48 h. After each time point (0, 3, 6, 9, 24, 48 h) a sample was taken, serially diluted using 0.1 % peptone and pour plated on TSA. Plates were incubated for 24 h at 37 °C and then colonies were enumerated.

5.3.7 Statistical Analysis

Results from the Hunter color tests and anthocyanin and phenolic tests were the mean value of three replicates ± standard error. Time kill assays were repeated twice and duplicate plating was used. Results were analyzed using SAS 9.3 (SAS, Cary, NC). A completely random design and ANOVA was used to find least significant differences ($p<0.05$).

5.4 Results and Discussion

The calyces from greenhouse-grown plants differed visually from the calyces that were acquired commercially. Calyces from 'Jamaican Cocktail Red' variety were more pink in color and had lower a-values and b-values and had a smaller calyx, whereas 'Thai Red' calyces were slightly larger in size and had a deeper red color or larger a-values and b-values (Table 5-1, Figure 5-1). The calyces from 'Roselle' were the most similar visually to the commercially available calyces and were deep red in color with a larger calyx and had higher a-values of ca. 48-49. A white commercially available variety, 'Saafara', was similar to the other commercial varieties except that it was white in color and free of anthocyanins and values for a were slightly negative and had higher values for L of around 70 (Table 5-1, Figure 5-1). After autoclaving, colors of the extracts all exhibited larger L-values and lower a-values and b-values (Table 5-1).

The anthocyanin and phenolic content varied depending on the variety; however, phenolic content remained similar after autoclaving while monomeric anthocyanin content decreased after autoclaving in all varieties (Table 5-2). The white calyx extract also did not contain any red anthocyanins, as expected. Christian and Jackson (2009) compared the composition of three varieties of *H. sabdariffa*, which included a traditional red, early bearing red, and white variety

and found that the white variety did not contain red anthocyanins, but all varieties contained phenolic compounds ranging in content from 4.73 to 23.12 mg GAE/g. These results are similar to our study, where phenolic content was 9.31 to 23.24 mg GAE/g. Christain and Jackson (2009) also analyzed for antioxidant activity and found that it ranged from 69 to 79% inhibition, which indicates extracts have a high capacity to prevent oxidation. The white variety also had high antioxidant activity and they attributed this to the high level of ascorbic acid present in the calyces (Christain and Jackson 2009). In their study they also examined different levels of maturity and determined that for the food industry the best variety to use was the early bearing red and harvest the calyces 35 d after flowering twice a year (Christain and Jackson 2009). In our study the greenhouse grown calyces were harvested approximately 2 wk after flowering to allow the plants to continue to flower.

All of the extracts used in this study demonstrated some level of antimicrobial activity against *S. aureus* ATCC 27708 (Figure 5-2). *S. aureus* was inactivated when grown in all extracts at 60 mg/ml (native pH 2.5) after 3 h. When the pH of the extracts was adjusted to 7, *S. aureus* was inhibited when grown in extract obtained from the commercial varieties as well as greenhouse-grown 'Roselle' at 60 mg/ml over 48 h. The other two greenhouse grown calyces ('Thai Red' and 'Jamaican Cocktail Red') were inhibitory up to 9 h but by 24 h, *S. aureus* had increased ca. 3 log CFU/ml. Although 'Thai Red' and 'Roselle'had similar levels of phenolic compounds (ca. 20 mg GAE/g), they differed in antimicrobial activity. Further work would need to be performed to determine the reason for this difference in activity. The pH of the extracts contributed to the antimicrobial activity, but was not the sole reason for activity. These results indicate that there is a potential synergistic effect among the compounds in the extracts. A recent study by Morales-

Cabrera and others (2013) examined five *Hibiscus sabdariffa* varieties. They tested ethanol, methanol, and aqueous extracts to determine the antimicrobial activity against *Salmonella.* The white variety 'Alma blanca' tested had the greatest antimicrobial activity and had the lowest pH when extracted with ethanol (pH 1.5). Phenolic compounds are known to be affected by pH.

5.5 Conclusions

In this study we compared the chemical composition and antimicrobial activity of 3 varieties of *H. sabdariffa* produced in the greenhouse to commercially acquired calyces. The greenhouse grown variety, Roselle, was most similar visually and chemically to the commercial varieties and demonstrated similar antimicrobial activity. Extracts from *Hibiscus* calyces could be used as a natural alternative to traditional antimicrobial preservatives to inhibit growth and/or eliminate pathogens in food products, especially in those products with a lower pH. The white commercially available variety, 'Saafara', was similar in antimicrobial activity to the other commercial varieties at pH 2.5 and was inhibitory at pH 7. Since this extract was free of anthocyanins, its use in foods may be more visually pleasing and an alternative to the bright red extract of the other varieties tested. Future work should include using chromatography techniques to identify the specific compound(s) responsible for antimicrobial activity as well as optimizing growth conditions of greenhouse grown varieties to maximize calyx production with the highest quantity of active compounds.

5.6 Acknowledgements

This research was supported by the Ivan Racheff Chair of Excellence endowment.

References

Chao CY, Yin MC. 2009. Antibacterial effects of roselle calyx extracts and protocatechuic acid in ground beef and apple juice. Foodborne Path Dis 6:201-6.

Christian KR, Jackson JC. 2009. Changes in total phenolic and monomeric anthocyanin composition and antioxidant activity of three varieties of sorrel (*Hibiscus sabdariffa*) during maturity. J Food Comp Anal 22: 663-7.

Cisse M, Dornier M, Sakho M, MarDiop C, Reynes M, Sock O. 2009. Bissap (*Hibiscus sabdariffa* L.) production in Senegal. Fruits. 64: 111-24.

Friedman M, Jurgens HS. 2000. Effect of pH on the stability of plant phenolic compounds. J Agric Food Chem 48:2101-10.

Hirunpanich V, Utaipat A, Morales NP, Bunyapraphatsara N, Sato H, Herunsalee A, Suthisisang C. 2005. Antioxidant effects of aqueous extracts from dried calyx of *Hibiscus sabdariffa* Linn. (roselle) *in vitro* using rat low-density lipoprotein (LDL). Biol Pharm Bull 28:481-4.

Liu K, Tsao S, Yin M. 2005. *In vitro* antibacterial activity of roselle calyx and protocatechuic acid. Phytother Res 19:942-5.

Morales-Cabrera M, Hernandez-Morales J, Leyva-Ruelas G, Salinas-Moreno Y, Soto-Rojas L, Castro-Rosas J. 2013. Influence of variety and extraction solvent on antibacterial activity of roselle (*Hibiscus sabdariffa* L.) calyces. J Med Plants Res 7:2319-22.

Morton J. 1987. Roselle. p. 281-286. In: Fruits of warm climates. Miami, FL. http://www.hort.purdue.edu/newcrop/Morton/roselle.html.

Ramierez-Rodrigues MM, Plaza ML, Azeredo A, Balaban MO, Marshall MR. 2011. Physiochemical and phytochemical properties of cold and hot water extraction from *Hibiscus sabdariffa*. J Food Sci 76:C428-35.

Appendix

Table 5- 1 L, a, b values ± SE of *Hibiscus sabdariffa* aqueous extracts (5 mg/ml) measured using Hunter colorimeter

Hibiscus variety	Filtered (4 °C, 0.22 µm membrane)			Autoclaved (121 °C, 30 min)		
	L	a	b	L	A	B
Roselle	43.89 ± 0.020	48.04 ± 0.041	24.34 ± 0.15	64.26 ±0.010	10.56 ± 0.0058	10.11 ± 0.041
Jamaican Cocktail Red	60.57 ± 0.0033	21.43 ± 0.0058	5.58 ± 0.032	69.04 ±0.66	1.11 ± 0.030	5.53 ± 0.078
Thai Red	42.95 ± 1.2	48.03 ± 0.92	27.64 ± 2.8	64.54 ± 0.062	9.88 ± 0.094	9.58 ± 0.062
Starwest Botanicals	38.73 ± 0.10	49.83 ±0.14	36.03 ± 0.49	66.69 ± 0.0088	5.89 ± 0.0058	9.75 ± 0.028
El Girasol	41.33 ± 0.040	48.37 ± 0.0088	26.84 ± 0.057	62.91 ± 0.11	10.36 ± 0.020	13.26 ± 0.0067
Saafara	70.59 ± 0.12	-1.85 ± 0.0058	7.79 ± 0.012	71.34 ± 0.0058	-2.68 ± 0.0066	8.69 ± 0.018

Table 5- 2 Anthocyanin and phenolic content of *Hibiscus sabdariffa* aqueous extracts.

Hibiscus Variety	Anthocyanin[a] Content (mg/L) ± SE		Phenolic[b] Content (mg/g) ± SE	
	Filtered (4 °C, 0.22 µm membrane)	Autoclaved (121 °C, 30 min)	Filtered (4 °C, 0.22 µm membrane)	Autoclaved (121 °C, 30 min)
Roselle	13.48 ± 0.47	0.57 ± 0.034	20.90 ± 0.65	20.50 ± 1.03
Jamaican Cocktail Red	1.93 ± 0.073	0.03 ± 0.017	10.53 ± 1.19	11.51 ± 0.07
Thai Red	10.84 ± 0.77	0.48 ± 0.16	20.84 ± 0.79	20.04 ± 0.72
Starwest Botanicals	8.38 ± 0.45	0.30 ± 0.05	16.90 ± 0.88	17.52 ± 0.30
El Girasol	15.61 ± 0.16	0.75 ± 0.076	23.24 ± 0.18	21.97 ± 0.83
Saafara	0 ± 0.00	0 ± 0.00	9.31 ± 0.26	11.88 ± 0.19

[a]Results expressed in cyanidin-3-glucoside equivalents, 520 nm

[b]Results expressed in gallic acid equivalents, 765 nm

Jamaican Cocktail Red Thai Red Roselle

Figure 5- 1. Calyces from three varieties of *Hibiscus sabdariffa* grown from seed.

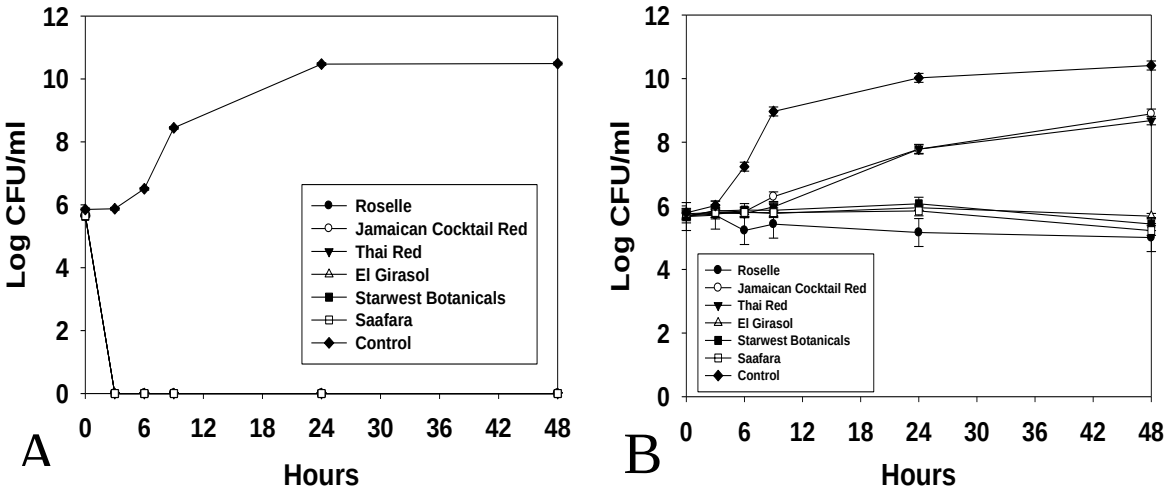

Figure 5- 2. Antimicrobial activity of *Hibiscus sabdariffa* extracts (commercial varieties: 'Starwest Botanicals', 'El Girasol' and 'Mi Costenita' and greenhouse grown varieties: 'Roselle', 'Jamaican Cocktail Red', and 'Thai Red') at a concentration of 60 mg/ml against *Staphylococcus aureus* ATCC 27708 in microbiological medium at native pH (A) and pH adjusted to 7 (B) after 48 h at 35-37 °C.

Chapter 6: Conclusions

Botanicals are excellent sources of bioactive compounds, which make them good candidates as natural antimicrobials. *Hibiscus sabdariffa* calyces have demonstrated to be effective antimicrobials against a variety of Gram-negative and Gram-positive foodborne pathogens. Extracts of *H. sabdariffa* calyces can be a natural alternative or adjunct to currently used synthetic antibiotics and are already accepted by the Food and Drug Administration as GRAS (generally recognized as safe).

This work has examined the activity of *Hibiscus* extracts in microbiological media and two food systems, milk and a rinse on hot dogs. Extracts demonstrated bactericidal effects when grown in milk at various fat levels against *Escherichia coli* O157:H7 and *Staphylococcus aureus*. Similarly, produced *Hibiscus* extracts were also effective as an antimicrobial rinse on hot dogs against *Listeria monocytogenes* and MRSA, with a greater effect against MRSA.

Hibiscus extracts were also relatively stable and enhanced antimicrobial activity was observed following autoclaving. This indicates that the extracts could be applied in the industry to foods that require further processing steps such as pasteurization or additional heating. However, extracts would be most effective in foods with acidic pH. When the pH was altered to 7 the extracts were less effective and lost some antimicrobial activity. The red color of the extracts along with their tart flavor makes them good options to be added to products such as juices. Further, these extracts also have the potential to be used as an antimicrobial rinse on ready-to-eat meat products such as hot dogs. Future work is needed to identify the specific compound(s) providing activity as well as consumer acceptance of flavor and color at active levels in foods.

Overall, *Hibiscus* extracts show great potential to be used in the food industry and could be utilized as a natural antimicrobial or preservative alone or in combination with other currently used antimicrobials.

Vita

Kristen Liane Higginbotham was born September 15, 1989 in Maryville, TN to John and Brenda (now Foley) Higginbotham. Kristen attended Knoxville Catholic High School and graduated with honors in 2008. She then began her undergraduate studies at the University of Tennessee, originally pursuing pharmacy. Once she discovered the Food Science program she knew she had found her calling. She enjoyed all of her classes, began an undergraduate research project, and graduated *Magna Cum Laude* with a Bachelor of Science degee in May 2012. Kristen then decided to pursue a Master of Science degree in Food Science and Technology that she began right after graduating in May 2012. She plans on pursuing a career in the food industry in research and development.